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(54) Title: EXPRESSION OF ALPHA-MACROGLOBULINS (57) Abstract <p>α-Macroglobulins, especially human α₂-macroglobulin, variants, fragments or derivatives thereof is produced by recombinant technology. The products are useful as additives to growth media, as proteinase inhibitors, as carrier in enzyme replacement therapy, and as DNA carrier in gene therapy.</p>		

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Title: Expression of alpha-macroglobulins

FIELD OF THE INVENTION

The present invention relates to the expression of α -macroglobulins, derivatives and variants thereof, and especially the expression of the human α_2 -macroglobulin (α_2 M) in an active form in mammalian cells, and the expression of genetically engineered variants thereof. The use of such recombinant α -macroglobulins, especially recombinant α_2 M ($r\alpha_2$ M) and variants is described with examples from the fields of medicine for therapeutic purposes, and the development of novel defined growth media for propagation of mammalian cells in culture.

BACKGROUND OF THE INVENTION.

BIOCHEMISTRY OF α_2 -MACROGLOBULIN (α_2 M).

15 The proteinase binding glycoprotein α_2 M, which is synthesized in the liver, constitute together with the complement proteins C3, C4 and C5 a separate class of structurally and functionally related large plasma proteins. For a recent review see (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, 20 FL).

Apart from C5 these proteins contain an internal β -cysteinyl- γ -glutamyl thiol ester, which enables the proteolytically activated forms of α_2 M, C3, and C4 to participate in characteristic covalent binding reactions (Sottrup-Jensen, L., et al., (1980) FEBS Lett. 121: 275-280; Salvesen, G.S. 25 and Barrett, A.J., (1981) Biochem. J. 187: 695-701). The thiol ester structure, which in the active proteins can be slowly cleaved by a number of small nitrogen nucleophiles, constitutes a unique type of postsynthetic modification of proteins, and plays a prominent role in the biological properties of α_2 M. The presence of the active thiol esters in α_2 M is revealed 30 by a characteristic pattern of heat fragmentation (Harpel, P.C., et al., (1979) J. Biol. Chem. 254: 8869-8878).

Traditionally, α_2 M has been studied within the context of plasma proteinase inhibitors, although by several criteria it is unique. Whereas most plasma proteinase inhibitors are monomeric proteins of roughly similar 35 size, containing approximately 430-500 residues, α_2 M is a tetramer whose 180-kD subunits contain 1451 residues (Sottrup-Jensen et al., (1984) J. Biol. Chem. 259: 8318-8327).

Furthermore, in contrast to most other proteinase inhibitors, which form 1:1 complexes with serine proteinases engaging the active site

of the proteinase and the reactive site of the inhibitor, α_2 M forms complexes with a broad spectrum of proteinases differing in their substrate specificity and catalytic mechanism e.g.: trypsin, leucocyte elastase, chymotrypsin, pancreatic elastase, cathepsin G, plasmin, plasma kallikrein and thrombin.

5 The second-order rate constant for association between these proteinases and α_2 M varies by several orders of magnitude. Both 1:1 and 2:1 proteinase- α_2 M complexes can be formed, and the disulfide-bridged dimer (360 kD) appears to be the functional unit of α_2 M (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, 10 Orlando, FL). Contrary to "classical" proteinase inhibitor complexes the α_2 M bound proteinase is still active, especially toward small synthetic substrates (Sottrup-Jensen, L. (1987) in: "The Plasma Proteins" (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL).

 The mechanism of proteinase binding by α_2 M has been described by 15 the "trap" (Barrett, A.J. and Starkey, P.M. (1973) Biochem. J. 133: 709-724), where proteolytic cleavage of a particularly exposed peptide stretch near the middle of the 180-kD subunit (the "bait" region) results in a conformational change of the α_2 M tetramer, thereby entrapping the proteinase. The nature of the essentially irreversible proteinase complex formation 20 with α_2 M has long remained elusive. However, recent investigations show that a major fraction (typically > 80-90 % of the trapped proteinase is also covalently bound through epsilon-lysyl(proteinase)- γ -glutamyl(α_2 M) bonds (Sottrup-Jensen, L. et al., (1981) FEBS Lett. 128: 127-132; Sand, O. et al., (1985) J. Biol. Chem. 260: 15723-15735; Pochon, F. et al., (1987) FEBS Lett. 217: 25 101-105).

PHYSIOLOGICAL ASPECTS OF PROTEINASE- α_2 M INTERACTIONS.

 Since the α_2 M-proteinase complexes are rapidly cleared from the circulation (Ohlsson, K. (1971) Acta Physiol. Scand. 81: 269-272; Imber, 30 M.J. and Pizzo, S.V. (1981) J. Biol. Chem. 256: 8134-8139.) a general role as a "clearing vehicle" for plasma proteinases has been envisaged.

 The main physiological targets may include proteinases of the coagulation and fibrinolysis systems and plasma kallikrein, and perhaps also proteinases like leucocyte elastase, cathepsin G and collagenases and other 35 proteinases released during cellular turnover (Sottrup-Jensen, L. and Birkedal-Hansen, H. (1989) J. Biol. Chem. 264: 393-401).

 Although α_2 M may be largely confined to the vasculature in healthy uninflamed tissues, the inhibitor and its proteinase complexes are found at near plasma levels in inflammatory exudates of rheumatoid joints and gingival

crevicular fluids (Tollefsen, T. and Saltved, E. (1980) J. Periodont. Res. 15: 96-106; Borth, W., et al., (1983) Ann. N. Y. Acad. Sci. 421: 377-381).

While plasma α_2 M appear to be synthesized in the liver (Schreiber, G. (1987) in: "The Plasma Proteins" (Putnam, F.W., ed) 2nd Ed., 5: 294-363, Academic Press, Orlando, FL.) other sites of synthesis exist. Several cell strains in culture have been shown to produce α_2 M including fibroblasts (Mosher, D.F., et al., (1977) J. Clin. Invest. 60: 1036-1045) and monocytes/macrophages (Hovi, T., et al., (1977) J. Exp. Med. 145: 1580-1589).

Whereas hepatocytes and Kupffer cells of the liver are most important for clearance of α_2 M-proteinase complexes in plasma (Davidsen, O., et al., (1985) Biochim. Biophys. Acta 846: 85-92), fibroblasts (Van Leuven, F., et al., (1979) J. Biol. Chem. 254: 5155-5160; Mosher, D.F. and Vaheri, A. (1980) Biochim. Biophys. Acta 627: 113-122) and macrophages (Debanne, M.T., et al., (1975) Biochim. Biophys. Acta 411: 295-304; Kaplan, J. and Nielsen, M.L. (1979) J. Biol. Chem. 254: 7323-7328) also possess receptors for α_2 M-proteinase complexes.

These observations suggest that there may be a considerable extravascular turnover of α_2 M perhaps primarily carrying proteinases functioning in the cellular micro environment (Sottrup-Jensen, L. and Birkedal-Hansen, H. (1989) J. Biol. Chem. 264: 393-401).

SUMMARY OF THE INVENTION

Briefly stated, the present invention discloses a method for the production of recombinant α -macroglobulins, and especially human α_2 M, and variants thereof in an active form.

Within a preferred embodiment, the cultured host cell is an eukaryotic cell such as a mammalian cell or cells derived from organisms such as insects, plants, yeast or other fungi, such as Aspergillus.

The invention further relates to DNA sequences comprising a gene encoding for the expression of human α_2 M and variants thereof, vectors comprising such DNA sequences, and suitable hosts transformed with such vectors.

Yet another aspect of the invention is the use of recombinant α_2 M and variants thereof as a protein carrier in enzyme replacement therapy (ERT).

Yet another aspect of the invention is the use of recombinant α_2 M and variants thereof as a DNA carrier in gene therapy.

Further aspects of the invention relates to the use of recombinant α -macroglobulins, especially human α_2 M, and variants thereof as

constituents of growth media, either as an additive or co-expressed with a desired gene product.

DEFINITIONS

5 Prior to setting forth the invention it may be helpful for an understanding thereof to set forth definitions of certain terms to be used hereafter.

Complementary DNA or cDNA: A DNA molecule or sequence which have been 10 enzymatically synthesized from sequences present in a mRNA template.

DNA Construct: A DNA molecule, or a clone of such a molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which 15 are combined and juxtaposed in a manner which would not otherwise exist in nature.

Plasmid or Vector: A DNA construct containing genetic information which may provide for its replication when inserted into a host cell. A plasmid 20 generally contains at least one gene sequence to be expressed in the host cell, as well as sequences encoding functions which facilitate such gene expression, including promoters and transcription initiation sites. It may be a linear or closed circular molecule.

25 **Joined:** DNA sequences are said to be joined when the 5' and 3' ends of one sequence are attached by phosphodiester bonds to the 3' and 5' ends, respectively, of an adjacent sequence. Joining may be achieved by such methods as ligation of blunt or cohesive termini, by synthesis of joined sequences through cDNA cloning, or by removal of intervening sequences 30 through a process of directed mutagenesis.

Variant: A peptide related to the original peptide, but wherein the amino acid sequence has been altered through mutation of the gene encoding the original peptide.

ABBREVIATIONSAMINO ACIDS

A	=	Ala	=	Alanine
V	=	Val	=	Valine
5 L	=	Leu	=	Leucine
I	=	Ile	=	Isoleucine
P	=	Pro	=	Proline
F	=	Phe	=	Phenylalanine
W	=	Trp	=	Tryptophan
10 M	=	Met	=	Methionine
G	=	Gly	=	Glycine
S	=	Ser	=	Serine
T	=	Thr	=	Threonine
C	=	Cys	=	Cysteine
15 Y	=	Tyr	=	Tyrosine
N	=	Asn	=	Asparagine
Q	=	Gln	=	Glutamine
D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid
20 K	=	Lys	=	Lysine
R	=	Arg	=	Arginine
H	=	His	=	Histidine

NUCLEIC ACID BASES

25 A	=	Adenine
G	=	Guanine
C	=	Cytosine
T	=	Thymine(only in DNA)
U	=	Uracil (only in RNA)

30

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a illustrates the construction of plasmid p1136.

Figure 1b illustrates the construction of plasmid p1167.

35 Figure 2 illustrates the structure of plasmid p1167.

Figure 3 illustrates a gel electrophoresis (10 - 20 % SDS-PAGE) of the thermal fragmentation products generated from α_2 M and α_2 M.

Figure 4 illustrates a gel electrophoresis of the thermal fragmentation products generated from methylamine treated α_2 M and α_2 M.

Figure 5 illustrates a gel electrophoresis (SDS-PAGE) of the reaction products generated from trypsin treatment of α_2 M and α_2 M.

Figure 6 illustrates a gel electrophoresis of the reaction products generated from trypsin treatment of methylamine-treated α_2 M and α_2 M.

5 Figure 7 illustrates a "rate gel" electrophoresis of unreacted native -and trypsin treated α_2 M and α_2 M.

Figure 8 illustrates a "rate gel" electrophoresis of unreacted native -and methylamine treated α_2 M and α_2 M.

Figure 9 illustrates the chromatograms of α_2 M and α_2 M on a 10 Superose 6 column.

Figure 10 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from chymotrypsin treated human α_2 M, human PZP and α_2 M-PZP.

Figure 11 illustrates the gel electrophoresis (10 - 20 % reducing 15 SDS-PAGE) of the reaction products from elastase treated human α_2 M, human PZP and α_2 M-PZP.

Figure 12 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from trypsin treated human α_2 M, human PZP and α_2 M-PZP.

20 Figure 13 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from Staphylococcus aureus Glu-specific protease treated human α_2 M, human PZP and α_2 M-PZP.

25 DETAILED DESCRIPTION OF THE INVENTION

According to the invention there is provided a process for the production of α -macroglobulins, especially human α_2 -macroglobulin, or fragments or derivatives, including variants thereof, wherein a functionally operative expression vector comprising a gene encoding for the expression of 30 a α -macroglobulin, especially human α_2 -macroglobulin, or fragments or derivatives thereof, including variants, or alleles of such a gene, is introduced into a suitable host capable of expressing said gene, said host is cultured in a suitable nutrient medium containing sources of assimilable carbon and nitrogen and other essential nutrients, and the expressed α - 35 macroglobulin, especially human α_2 -macroglobulin, or fragments or derivatives thereof is recovered.

Many proteins synthesized particularly in mammalian cells undergo post-translational modification (processing) of one kind or the other.

Depending on the final destination and on the specific function of a newly synthesized protein, it may go through a number of processing steps leading to covalent modifications such as e.g.: glycosylation, γ -carboxylation, β -hydroxylation, sulphatation, amidation, thiol ester formation, phosphorylation, proteolytic cleavage at precursor processing sites, fatty acylation (Rosner, M.R. (1986). in: "Mammalian Cell Technology", (Thilly, W.G. ed), Butterworth Publishers, Stoneham, MA.: 63-89).

Proteins of various sizes and with a variety of different post-translational modifications have been successfully expressed in transformed heterologous mammalian host cells using recombinant DNA technology. A few examples: Human coagulation factors VIIa and IX have been expressed in transformed BHK (Syrian Baby Hamster Kidney) cells with correct post-translational modifications such as γ -carboxylation and glycosylation (Thim, L. et al., (1988) *Biochemistry* 27: 7785-7793; Busby, S. et al., (1985) *Nature* 316: 271-273). Human Platelet-derived Growth Factor AB heterodimer has been expressed in transformed CHO (Chinese Hamster Ovary) cells with correct processing of the A and B chain precursors and correct assembly of the AB heterodimer. Human coagulation factor VIII has been expressed in transformed CHO cells with correct processing of the precursor leading to a two chain molecule that can be activated by thrombin and factor Xa (Kaufman, R.J. et al., (1988) *J. Biol. Chem.* 263: 6352-6362; Pittman, D.D. and Kaufman, R.J. (1988) *Proc. Natl. Acad. Sci. USA* 85: 2429-2433).

So far, there have been no reports on the heterologous expression of proteins in which the formation of an active thiol ester is a prominent post-translational modification.

The biosynthesis of the internal thiol ester in the third component (C3) of complement from rabbit has been investigated (Iijima, M. et al., (1984) *J. Biochem.* 96: 1539-1546). Rabbit liver mRNA was translated in vitro in a rabbit reticulocyte lysate system, and the synthesized C3 specific products did not incorporate radio labelled methylamine. On the other hand radio labelled iodoacetamide reacted with the synthesized C3 specific products; these results indicated the presence in the primary C3 specific translation product of a free thiol group instead of a reactive thiol ester. If a liver homogenate supernatant (S-13) including cytosol and microsomes was included, the C3 specific product could now incorporate methylamine. By increasing the concentration of the S-13 component(s), the incorporation of methylamine in C3 specific products was increased, and at the same time incorporation of iodoacetamide decreased. If the S-13 fraction was treated at 65°C for 5 min, the activity was completely lost.

The results from this investigation strongly suggest an involvement of a transglutaminase-like or other type of enzyme in the posttranslational formation of an active thiol ester in rabbit C3. There are no similar investigations addressing the formation of the thiol ester in other α -macroglobulins, e.g. α_2 M, but from analogy and homology considerations, it is expected that a similar mechanism is responsible for the formation of thiol esters in other α -macroglobulins synthesized in the mammalian liver.

Through this investigation a number of developments were done 10 which also are deemed to be encompassed of the present invention. These include DNA sequences comprising a gene encoding for the expression of α -macroglobulins, especially human α_2 -macroglobulin, or fragments or derivatives and variants thereof as exemplified in SEQ ID NO:1 and SEQ ID NO:3.

Another aspect of the invention relates to functionally operative 15 expression vectors comprising a gene encoding for the expression of at least one α -macroglobulin, especially human α_2 -macroglobulin or fragments or derivatives and variants thereof, or alleles of such a gene.

Such vectors preferably further comprise regulatory elements necessary for the stable maintenance of said vector in mammalian cells.

20 Also, such vectors may further include sequences providing for the processing and secretion of the expressed product.

In relation to the use of recombinant α -macroglobulins, and especially α_2 M, in growth media it may be co-expressed with another desired gene product, and consequently the vectors of the invention may further 25 comprise one or more other genes encoding for a desired gene product.

The invention further relates to transformed hosts comprising a functionally operative expression vector according to the invention comprising a gene encoding for the expression of human α_2 -macroglobulin or fragments 30 or derivatives and variants thereof, or alleles of such a gene.

The host may be selected from the group comprising a bacterial strain, a fungal strain, a mammalian cell line, or a mammal, especially a fungus, such as belonging to the genus Aspergillus, or a yeast strain, preferably belonging to the genus Saccharomyces.

35 Another preferred type of host is a mammalian cell line, preferably a Syrian Baby Hamster Kidney (BHK) cell line, and especially the one which is available from ATCC under No. CRL 1632.

The invention further relates to the recombinant human α_2 -macroglobulin or a variant thereof in an active form having the amino acid sequence of SEQ ID NO:2, or SEQ ID NO:4.

5 APPLICATIONS OF α -MACROGLOBULINS, ESPECIALLY α_2 M.

The present invention discloses applications of α -macroglobulins, and especially α_2 M. These should be regarded not as limitations but as a few examples among many for the use of recombinant derived α -macroglobulins.

10 α -MACROGLOBULINS AS CONSTITUENTS OF DEFINED GROWTH MEDIA.

Degradation of specific heterologous products produced in either transformed or non-transformed mammalian cells is a potential problem in the production of recombinant products. This is due to the fact that many host cells secrete one or more different proteinases.

15 When a production cell line is grown in the presence of e.g. 10 % fetal calf serum, such proteolytic degradation of secreted recombinant or native protein products is a minor problem due to a buffering effect of the added serum proteins.

However, the use of fetal calf serum in the large scale growth
20 (fermentation) of mammalian production cell lines is not a desirable situation for a number of reasons. First of all fetal calf serum is a very costly constituent of complex growth media; second, the demand for fetal calf serum from a growing biopharmaceutical industry might not be easily fulfilled in the future, and third, the use of fetal calf serum constitutes
25 a potential quality control problem in the production of pharmaceuticals intended for use in humans.

To circumvent these problems, efforts can be expected in the field of development of defined growth media for use with mammalian cells.

Addition of various proteinase inhibitors to such new defined
30 growth media will be required to ensure the integrity of the secreted products. Alternatively, the producer cell line might, through genetic engineering, be endowed with the capacity to produce and secrete proteinase inhibitors along with the desired product(s).

α -Macroglobulins, and especially Human α_2 M, are proteinase
35 inhibitors of broad specificity, and they are therefore according to the invention used as constituents of defined growth media for mammalian cells, either as a medium additive or as a product co-produced with the desired product.

The target sites for a number of different proteinases, e.g. bovine trypsin, Streptomyces griseus trypsin, papain, porcine elastase, bovine chymosin, bovine chymotrypsin, Staphylococcus aureus strain V8 proteinase, human plasmin, bovine thrombin, thermolysin, subtilisin Novo and 5 Streptomyces griseus proteinase B have been mapped in the bait region of human α_2 M (Mortensen, S.B., et al., (1981) FEBS Lett. 135: 295-300) and other α -macroglobulins (Sottrup-Jensen, L., Sand, O., Kristensen, L. and Fey, G.H. J.Biol.Chem. 264,15781-15789, 1989). It is evident that α_2 M and the other α -macroglobulins as proteinase inhibitors have broad specificities.

10 In those situations, where the proteinase inhibitory spectrum of a α -macroglobulin, such as α_2 M, is not sufficient for the prevention of product degradation, it is possible through site specific mutation, protein engineering, etc. to change the proteinase inhibitor specificity of the α -macroglobulin, such as α_2 M. Incorporation of desirable specific proteinase 15 target sites in the bait region of recombinant α_2 M will change the inhibitor specificity of the mutated α_2 M. Furthermore it is possible through genetic engineering to construct novel specific or general proteinase target sites in the bait region of a α -macroglobulin in order to enhance its versatility as a proteinase inhibitor of specific or broad inhibitory spectrum. 20 Furthermore it is possible to remove specific target sites in an α -macroglobulin in order to avoid degradation of the variant in question by certain proteases in the circulation that will already be inhibited through the action of naturally present proteinase inhibitors.

The production of recombinant products in fungi, such as species 25 and strains of e.g. Aspergillus and Saccharomyces also meets with potential problems of product degradation. In some cases it is possible to isolate proteinase negative mutants of desirable production strains. This might not always be the case, and co-expression of α -macroglobulins, such as α_2 M or α_2 M-mutants together with a desirable product may inhibit proteolysis of the 30 product in question.

α -MACROGLOBULIN MUTANTS AS SPECIFIC PROTEINASE INHIBITORS.

The amino acid sequence of the bait region of α -macroglobulins defines the specificity of the α -macroglobulin towards different proteina- 35 ses. A comparison of cleavage patterns for different proteinases and bait region sequences in five mammalian α -macroglobulins has recently been published (Sottrup-Jensen, L., Sand, O., Kristensen, L. and Fey, G.H. The α -macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian α -macroglobulins. J. Biol. Chem. 264,

15781-15789, 1989). It has previously been clearly demonstrated that the bait region in each species of α -macroglobulin is the major determinant of proteinase inhibitor specificity. The present invention demonstrates the possibility of modulating the inhibitor specificity of human α_2 M by 5 alterations of proteinase target sites in the bait region.

In the present invention it is demonstrated that the bait region of human α_2 M (residues 690 to 730 in SEQ ID NO:2) can be mutated at will to obtain a new proteinase inhibitor profile of this macroglobulin. The example presented in the present invention describes the construction of a hybrid 10 macroglobulin. In this hybrid the bait region from human pregnancy zone protein (PZP) was introduced into human α_2 M, from which the native bait region had been removed. The hybrid molecule, which was constructed by the use of recombinant DNA technology, revealed a proteinase inhibitor profile similar to the inhibitor profile of PZP.

15 The invention thus demonstrates the possibility to design and produce proteinase inhibitors with altered and new inhibitor specificities at will.

This finding is important for the design of new proteinase inhibitors. Due to the low antigenicity the bait region in macroglobulins 20 (Van Leuven, F., Marynen, P., Cassiman, J.-J. and Van den Berghe, H. Mapping of structure-function relationships in proteins with a panel of monoclonal antibodies. A study on human alpha-2-macroglobulin. J. Immunol. Methods 111, 39-49, 1988, and Delain, E., Barray, M., Tapon-Brethaudiere, J., Pochon, F., Marynen, P., Cassiman, J.-J., Van den Berghe, H. and Van Leuven, F. The 25 Molecular Organization of Human alpha2-Macroglobulin. An Immunoelectron microscopic study with monoclonal antibodies. J. Biol. Chem. 263, 2981-2989, 1988) it is now possible, by the use of the technology described in the present invention, to design non-immunogenic new proteinase inhibitors that can be used e.g. in the treatment of any disease, where aggressive proteina- 30 ses constitute a threat to the health of man.

In the present specification the production of α_2 M variants is described by the construction of a hybrid macroglobulin. It is clear to the skilled person in the art that changes also could be obtained through other genetic engineering methods, such as described in International Publication 35 No. WO 89/06279 (NOVO INDUSTRI A/S). Also it is clear that other α -macroglobulins could be employed instead of the human α_2 M, such as those mentioned in Sottrup-Jensen, L. et al. (1989), supra.

α_2 M AS A PROTEIN CARRIER IN ENZYME REPLACEMENT THERAPY.

A different application of α_2 M is its use as a carrier of macromolecules such as proteins and nucleic acids. When α_2 M reacts with and forms a complex with a proteinase in solution, α_2 M may bind other proteins (also non-proteinase proteins) present in that solution (Salvesen, G.S. et al., (1981) Biochem. J. 195: 453-461). In the case of Fabry's disease, which is an X-chromosome linked disorder of glycosphingolipid metabolism, it has recently been demonstrated that α_2 M can function as a carrier in an in vitro model of enzyme replacement therapy (ERT) (Osada, T., et al., (1987) Biochem. Biophys. Res. Commu. 142: 100-106). α_2 M was conjugated to coffee bean α -galactosidase through the action of trypsin, and the formed complex was internalized through α_2 M-receptor specific (Van Leuven, F., et al., (1981) J. Biol. Chem. 256: 9016-9022) endocytosis and delivered to the lysosomes, which is the target organelle for α_2 M-receptor mediated internalization of α_2 M-proteinase complexes (Willingham, M.C. and Pastan, I., (1980) Cell 21: 67-77).

Such a scheme in ERT provides a method of internalization to the lysosome of the enzyme in question and at the same time it might alleviate potential antigenicity problems arising from the use of heterologous enzymes in therapy. One limitation in this type of ERT (Osada, T., et al., (1987) Biochem. Biophys. Res. Commu. 142: 100-106) would be the types of potential target cells that could be treated by this protocol. Obviously, they would have to express the α_2 M-receptor. In a future development of the system, the possibility might exist to redesign the cell specificity of α_2 M internalization by exchanging the receptor binding domain of α_2 M with other receptor ligands. Hereby α_2 M-mutants could be designed to enter any cell type known to express a specific internalizable receptor.

This type of development would of course require a system for the production of recombinant derived α_2 M. The use of native human α_2 M as a carrier in ERT (as described above) is undesirable due to the now well known risks of the employment of blood derived products in the treatment of human disease.

The production of recombinant α_2 M in accordance with the present invention alleviates this problem by providing for large scale production of α_2 M.

 α_2 M AS A DNA CARRIER IN GENE THERAPY.

Advances in gene transfer into mammalian cells have opened for the possibility of the treatment of a number of genetic disorders through

gene therapy. A major problem in gene therapy will be the specific targeting of genes into the appropriate cells within the body. (Williamson, B., (1982) *Nature* 298: 416-418; Anderson, W.F., (1984) *Science* 226: 401-409; Parkman, R., (1986) *Science* 232: 1373-1378).

5 It was recently described that a constructed foreign gene containing the chloramphenicol acetyltransferase (CAT) on a bacterial plasmid could be targeted to the liver of rats by specific receptor directed internalization (Wu, G.Y. and Wu, C.H. (1988) *J. Biol. Chem.* 263: 14621-14624). The DNA carrier consisted of a galactose-terminal (asialo)glyco-
10 protein and asialoorosomucoid covalently linked to poly-L-lysine. The polycation poly-L-lysine can bind DNA in a strong non-covalent and nondamaging interaction. It was demonstrated that complex bound DNA was internalized by cell-surface asialoglycoprotein receptors that are unique to hepatocytes. The complex was injected intravenously, and upon analysis only the liver
15 expressed the CAT activity.

In the present invention the use of α_2M as a carrier of DNA in gene therapy is suggested. Reaction of α_2M with a proteinase such as trypsin or with methylamine in the presence of covalently closed circular plasmid DNA is likely to result in partial or total entrapment of DNA within the
20 complexing α_2M molecule. After intravenous injection of such complexes with exposed receptor binding domains, the complex will be rapidly cleared from the blood and internalized in specific target cells, such as hepatocytes and Kupffer cells. Through protein engineering on the receptor binding domain of α_2M it will be possible to design a DNA carrier specific for other cell
25 types. The advantage in this system as compared to the above described system using the asialoglycoprotein receptor is, that it will not be necessary to identify different DNA carrier systems for each new cell type.

30 EXAMPLES

Materials and methods:

Microorganisms and cell lines

E. coli K12 (MC1061) is available from e.g. Stratagene Inc.,
35 11099 North Torrey Pines Rd., La Jolla, California 92037.

HepG2 (Human hepatoblastoma cell line) is freely available from American Type Culture Collection, under No. HB 8065.

BHK (Syrian Hamster Kidney cell line, thymidine kinase mutant line tk⁻sl3, (Waechter and Baserga (1982) *Proc. Natl. Acad. Sci. USA* 79:

1106-1110); is freely available from American Type Culture Collection, under No. CRL 1632.

Plasmids and vectors

5

Plasmids pCDVI-PL and pSP62-K2 are available from Dr. Tasuku Honjo, Faculty of Medicine, Kyoto University, Kyoto 606, Japan. pSP62-K2 was derived from the plasmid pSP62-PL (available from New England Nuclear/Du Pont (U.K.) Ltd., Wedgwood Way, Stevenage, Hertfordshire, SG14QN) as described (Noma et al., (1986) Nature, 319: 640-646). pCDVI-PL was derived from pcDV1 (Okayama, H. and Berg, P. (1983) Molec. cell. Biol. 3: 280-289) as described (Noma et al., (1986) Nature, 319: 640-646).

M13mpl8 is available from Pharmacia LKB Biotechnology (catalog # 27-1552-01) (Norlander, J., Kempe, T. and Messing, J. Gene 26: 101-106, 15 1983).

M13mpl9 is available from e.g. International Biotechnologies, Inc., P.O. Box 9558, 275 Winchester Avenue, New Haven, Connecticut 06535, USA.

pDHFR-I is available from Dr. K.L. Berkner, ZymoGenetics Inc., 20 4225 Roosevelt Way NE, Seattle, Washington 98105. (The construction of this plasmid is given in detail in: Berkner, K.L. and Sharp, P.A. (1984) Nucleic Acids Res. 12: 1925-1941). The molecular cloning of the DHFR cDNA present in this plasmid, and its sub-cloning in mammalian expression vectors under the control of adenovirus derived promoters has previously been described 25 in detail (Chang, A.C.Y., et al., Nature 275: 617-624 and Kaufman, R.J. and Sharp, P.A. (1982) Mol. Cell. Biol. 2: 1304-1319). The backbone plasmid in pDHFR-I is pBR322 (Sutcliffe, J.G. (1979) Cold Spring Harbor Symp. Quant. Biol. 43: 77-90; Sutcliffe, J.G. (1978) Nucleic. Acids Res. 5: 2721-2728).

pUC13 is described in: Vieira, J. and Messing, J.: 1982, Gene 19: 30 259-268 and available from Pharmacia LKB Biotechnology (catalog # 27-4954-01).

pUC19 is described in: Yanisch-Perron, C. and Messing, J., 1985, Gene 33:103-119 and available from Pharmacia LKB Biotechnology (catalog # 27-4951-01).

35

Growth media**LB-broth:**

Mix 227 g Bacto Tryptone, Difco 0123-01
 113.5 g Yeast extract, Difco 0127-01, and
5 227 g NaCl in a sealable plastic container.

Add 12.5 g mix to 500 ml water in a 1000 ml bottle, shake well and sterilize in an autoclave.

Dulbeccos Modified Eagle Medium is available from e.g. Gibco Ltd.
10 P.O. Box 35, Trident House, Renfrew Road, Paisley PA34EF, Renfrewshire,
Scotland. Cat.# 042-250 1M (10 * concentrate).

Antibodies

15 Anti- α_2 M A033 and peroxidase conjugated anti- α_2 M PE326 were from
DAKOPATTS A/S, Copenhagen, Denmark.

EXAMPLE 1.**CLONING AND SEQUENCE DETERMINATION OF HUMAN α_2 M**

20

Preparation of messenger RNA from the human cell line HepG2.

The human hepatoblastoma cell line HepG2 (American Type Culture Collection No. HB 8065, freely available) was used as a source for mRNA preparation. HepG2 cells were grown to a total cell number of 15×10^7 in
25 Dulbecco's Modified Eagle medium containing 10% fetal calf serum and antibiotics.

Total RNA was isolated by the guanidinium thiocyanate method (Chirgwin et al., (1979) Biochemistry 18: 5293-5299) and purified by CsCl gradient centrifugation. A total of 3000 μ g RNA was obtained. mRNA was
30 isolated by use of an oligo(dT)-cellulose column (Aviv & Leder (1972) Proc. Natl. Acad. Sci. USA 69: 1408-1412). 60 μ g of mRNA was obtained after one cycle of affinity chromatography. After ethanol precipitation, this preparation of mRNA was resuspended in 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA- Na_2 at a final concentration of 1 μ g/ μ l and stored at -80°C for subsequent
35 use in the construction of a cDNA library.

Construction of a cDNA library from HepG2 mRNA.

A cDNA library was constructed in the pCDVI-PL/pSP62-K2 vectors (Noma et al., (1986) Nature, 319: 640-646. Available from Dr. Tasuku Honjo,

Faculty of Medicine, Kyoto University, Kyoto 606, Japan) by use of the methods described by Okayama & Berg (Mol. Cell. Biol. 2: 161-170 (1982); Mol. Cell. Biol. 3: 280-289 (1983)).

E. coli K12 (MC1061) (Casadaban & Cohen (1980) J. Mol. Biol. 138: 179-207) was used for transformation. MC1061 were grown in L-broth at 37°C to OD₆₀₀=0.5. Twenty ml were centrifuged, and the pellet was resuspended in 7 ml of ice-cold sterile 0.1 M CaCl₂, incubated on ice for 30 minutes, centrifuged briefly, and finally kept in the cold room overnight.

Ninety-five µl suspension of transformation-competent E. coli MC1061 were added per 10 µl of cDNA preparation. The mixture was incubated on ice for 30 minutes, heat-shocked at 43.5°C for 45 seconds, and finally, after addition of L-broth, incubated at 37°C for 30 minutes.

After resuspension, the cells were plated onto L-broth plates containing ampicillin (50 µg/ml) and grown for 8 hrs at 37°C. A total of 2.9 $\times 10^5$ individual colonies could be obtained from this library.

Screening of the HepG2 library for cDNA clones encoding human α_2 M.

5 $\times 10^4$ individual colonies were screened by standard colony hybridization technique using nitrocellulose filters (Maniatis et al., (1982) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, New York).

A 20-mer oligonucleotide mixture

5' CC(T/C)TTCAT(G/A)TC(T/C)TC(T/C)TG(T/C)TT 3'

where the notation (X/Y) means that either of the nucleic acids X or Y may be used, complementary to the human α_2 M mRNA in the region encoding amino acid residues Lys-Gln-Glu-Asp-Met-Lys-Gly (residues number 493 - 499 in Sottrup-Jensen et al., J. Biol. Chem. 259: 8318-8327 (1984) was synthesized (on a DNA synthesizer from Applied Biosystems, USA), labelled with ³²P (using T₄ polynucleotide kinase and γ -³²P-ATP) to a specific activity of 3 $\times 10^6$ cpm/pmol oligonucleotide. The labelled oligonucleotides were purified by gel chromatography and subsequently used in the screening of the cDNA library.

The hybridization solution contained 6 \times SSC, 5 \times Denhardt's solution, 0.05% SDS (Maniatis et al., (1982) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, New York) and 10⁶ cpm/ml of labelled oligonucleotide mix.

35 Hybridization was performed for 3 hrs at 45°C. Then the filters were washed in 6 \times SSC, 0.05% SDS at 45°C for 3 \times 10 minutes. After autoradiography the filters were washed under the same conditions, but this time at 52°C. A colony that still showed hybridization at this temperature was isolated and the cDNA insert of the corresponding plasmid (designated p α_2 M)

from this isolate was sequenced (Tabor & Richardson (1987) Proc. Natl. Acad. Sci. USA 84: 4767-4771). The sequence of the cDNA and the derived encoded amino acid sequence are shown in the appended sequence listings, SEQ ID NO:1:, and SEQ ID NO:2:.

5

Characterization of p α_2 M.

p α_2 M had a cDNA insert of approximately 4.6 kb. Its sequence is given in Table I above.

10 The sequence in Table I demonstrates that the entire coding region of α_2 M including the signal peptide is found in the insert.

In addition to the coding region, the insert contains sequences derived from the 5'- and 3' untranslated regions of the α_2 M mRNA molecule.

The amino acid sequence of the human α_2 M as deduced from the cDNA 15 in p α_2 M is in total agreement with the published sequence (Sottrup-Jensen et al., (1984) J. Biol. Chem. 259: 8318-8327). Codon number 1000 (numbered from the initiating methionine codon in the signal peptide) was found to be ATC encoding an isoleucine and not GTC (encoding a valine) as found in an α_2 M cDNA synthesized from human liver mRNA (Kan et al., (1985) Proc. Natl. Acad. Sci. 20 USA. 82: 2282-2286). In the α_2 M cDNA sequence from the HepG2 library we have further identified ten silent changes as compared to the sequence from the liver library, see the following Table I:

TABLE I

	Codon	Liver	HepG2
5	413 (Asn)	AAC	AAT
	495 (Phe)	TTT	TTC
10	750 (Gly)	GGG	GGT
	796 (Leu)	CTT	CTC
15	835 (Leu)	CTT	CTA
	1266 (Ala)	GCC	GCA
	1296 (Asn)	AAT	AAC
20	1326 (Thr)	ACC	ACA
	1442 (Leu)	CTC	CTG
25	1460 (Ile)	ATC	ATT

The position of the oligonucleotide mixture used as a hybridization probe in the colony screenings was from position 1574 to position 1594, and the position of the reactive thiol ester is from position 2939 to 2953 in SEQ ID NO:1.

EXAMPLE 2.

Construction of a mammalian expression vector for α_2 M.

α_2 M was digested (fig. 1a) with XbaI and EcoRI, and a 1.2 kb fragment containing the 5' part of the α_2 M cDNA together with the multiple cloning site of pSP62-K2 was isolated on an agarose gel and cloned in an XbaI/EcoRI digested M13mp19 vector to generate M13mp19A. To facilitate further subclonings of the α_2 M cDNA, a unique EcoRV site was introduced in the 1.2 kb fragment 10 nucleotides 5' to the initiating ATG (methionine) codon through site directed mutagenesis (Kunkel et al., (1987) Methods Enzymol. 154: 367-382). In the same mutagenesis experiment, in which the mutagenic oligonucleotide NOR593:

5'(TTCTTCCCATGGTGGATATCGAAGGAGCTG)3'

was used, the 5 nucleotides 5' to the methionine codon was changed to CCACCATG; this mutation creates a new NcoI site spanning the ATG codon. A

correct mutant M13mpl9B was identified through restriction enzyme digestion and DNA sequencing.

The mutated 5' end of α_2M cDNA was isolated from M13mpl9A replicative form through digestion with HindIII and EcoRI and agarose gel electrophoresis. The isolated DNA fragment was then joined to HindIII/EcoRI digested p α_2M through ligation to generate p1136. In this plasmid the α_2M cDNA is reassembled in its total length, but now with a unique EcoRV site at the 5' end. p1136 was digested with EcoRV/DraI, and the α_2M fragment was isolated on an agarose gel and cloned in a mammalian expression vector under control of the adenovirus 2 major late promoter (Ad 2 MLP).

The adenovirus-promoter based vector was constructed by K.L.Berkner (ZymoGenetics Inc., Seattle, WA.), and a detailed description of the functional elements in the mammalian expression vector is given in: Powell, J.S. et al., (1986) Proc. Natl. Acad. Sci. USA 83: 6465-6469 and in: Boel et al., (1987) FEBS Lett. 219: 181-188).

The expression vector used for expression of human α_2M was generated from the mammalian expression vector pPP (Boel, E. et al., (1987) FEBS Lett. 219: 181-188), in which human pancreatic polypeptide cDNA was cloned under control of Ad 2 MLP.

20 pPP was digested (fig. 1b) with BamHI and the resulting staggered ends were repaired with DNA polymerase (Klenow fragment and the four deoxynucleotide triphosphates). The 4.5 kb EcoRV/DraI α_2M cDNA fragment was joined to this vector through ligation, and correct recombinants were characterized through restriction enzyme analysis on isolated miniprep. 25 plasmids.

The α_2M -mRNA transcribed from the resulting 8.76 kb plasmid (designated p1167 (fig. 2)) has the adenovirus 2 late tripartite leader (L1-3) at its 5' end together with an mRNA splice signal (SS). At the 3' end of the construct the transcript is terminated with the SV40 late termination - 30 and polyadenylation signal. 5' to the Ad 2 MLP the construct includes the SV40 enhancer (ENH) and the 0 to 1 (0 - 1) map units from adenovirus 5.

Expression of α_2M in mammalian cells.

For expression of human α_2M in cultured BHK cells (Syrian Hamster 35 Kidney, thymidine kinase mutant line tk⁻sl3, (Waechter and Baserga (1982) Proc. Natl. Acad. Sci. USA 79: 1106-1110); American Type Culture Collection CRL 1632) the expression vector p1167 was co-transfected with pDHFR-I (Berkner, K.L. and Sharp, P.A. (1984) Nucleic Acids Res. 12: 1925-1941. Available from K.L.Berkner, ZymoGenetics Inc. Seattle) into subconfluent cells by the

calcium phosphate mediated transfection procedure (Graham and Van der Eb (1973) Virology 52: 456-467). In the transfection experiment the molar ratio between p1167 and pDHFR-I was 10:1. Cells were grown in Dulbeccos Modified Eagle Medium supplemented with 10% fetal calf serum (FCS).

5 Forty-eight hours after transfection, cells were trypsinized and diluted into medium containing 400 nM methotrexate (MTX). After 10 to 12 days, individual colonies were cloned out and expanded separately. The expanded cultures were propagated for 24 hours as described above, and producer clones were identified using an enzyme linked immunosorbent assays 10 (ELISA) (Munck Petersen C., et al., (1985) Scand. J. Clin. Lab. Invest. 45: 735-740) against human α_2 M secreted to the growth medium.

Description of the α_2 M ELISA assay.

The materials used in the ELISA were:

15 Catching antibody A033 anti- α_2 M,
 Peroxidase-conjugated anti- α_2 M antibody PE326,
 1,2-Phenylenediamine, dihydrochloride (OPD)
 all from DAKOPATTS A/S, Copenhagen, Denmark.
 Urea peroxide, 125 mg, was from Organon Teknika.
20 96 well ELISA plates were from NUNC, Copenhagen.

Coating buffer:

100 mM carbonate buffer pH 9.6 was made up as follows:
Add 3.18 g Na_2CO_3 and 5.96 g NaHCO_3 to 1000 ml water.

25

Standard and sample buffer:

To 100 ml of 150 mM phosphate buffer pH 7.2 was added:
50 μ l Tween 20
2 g Bovine Serum Albumin (Sigma A 7030).

30

Washing buffer:

10 mM sodium phosphate pH 7.4
145 mM sodium chloride
0.1 % Tween 20.

35

Citric acid-phosphate buffer, pH 4.9:

The following reagents were added to 1000 ml of water
7.3 g citric acid
23.88 g Na_2HPO_4 , 12 H_2O

0.5 ml Tween 20

The buffer was used for a maximum of 14 days, stored at 4°C.

Urea peroxide solution:

5 125 mg urea peroxide was dissolved in 8.93 ml water.

The solution was kept in the dark at 4°C.

Coating of the plates for assay:

The 96 well plate was coated with 175 µl of the DAKO A033
10 antibody diluted 1:1000 in the coating buffer. The plate was incubated over
night at 4°C. Before use the plate was washed 4 times in washing buffer.

Application of standards and samples:

100 µl standard or sample was added to each well. As a standard
15 purified human α_2 M, 2 mg/ml (prepared as described in: Sottrup-Jensen et al.,
(1983) Ann. N.Y. Acad. Sci. 421: 41-60) was used. The standard curve included
the following serial dilutions: 1:4000, 1:8000, 1:16000 etc. down to
1:1024000, corresponding to final concentrations from 500 µg/l down to 1.95
µg/l. All dilutions were done in the Standard and sample buffer. The plate
20 was incubated over night at 4°C and then washed 4 times with wash buffer
before the next step.

Addition of conjugated antibody:

100 µl of PE326, which had been diluted 1:6000 in the Standard
25 and sample buffer, was added to each well. The plate was incubated for 2 h
at 20°C, and then washed 4 times with wash buffer.

Enzyme activation:

8 mg of OPD was dissolved in 12 ml of Citric acid- phosphate
30 buffer. To this solution 500 µl Urea peroxide solution was added and the
mixture was used immediately. 100 µl of the final solution was added to each
well, and the plate was incubated in the dark for 6 min. Then 100 µl of 2 M
H₂SO₄ was added to each well and the A₄₉₂ was read in an automated ELISA plate
reader.

35

The above described ELISA did not give any background on medium
supplemented with 10% FCS, nor did it give any background in BHK cell
conditioned medium. Of 24 isolated MTX resistant clones, 16 produced
detectable amounts of recombinant α_2 M.

Selected cell lines that secreted 12.3 mg/l (K16-6) and 19.1 mg/l (K17-6) in the supernatant (grown in a 6 well NUNC-plate) over a 48 hour period were expanded for large scale production of recombinant human α_2 M (α_2 M).

5

Purification of recombinant human α_2 M.

Cell lines K16-6 and K17-6 were each expanded into one ten-double tray (NUNC, Denmark) with a growth surface of 6000 cm². At 80% confluency the medium on the cells was changed from containing the 10% fetal calf serum (FCS) down to 2%. After 48 hours of growth in medium with only 2% (FCS), the medium was removed, and the cells were washed twice with serum free medium. Cells were then grown serum free for 4 to 5 days with change of serum free medium every two days. Conditioned medium was pooled and analyzed for α_2 M by ELISA.

15 The pooled conditioned medium from K16-6 and from K17-6 contained 7.15 mg/l and 21.5 mg/l of α_2 M, respectively.

The α_2 M was purified according to published procedures (Sottrup-Jensen et al., (1983) Ann. N. Y. Acad. Sci. 421: 41-60). Briefly the conditioned medium was loaded onto a 10 ml Zn-Chelate column (Zn²⁺-iminodiacetic acid Sepharose 4B (Porath, J. et al., (1975) Nature 258: 598-599) equilibrated with 25 mM Tris-HCl pH 8.0, and washed with 100 ml phosphate buffered saline (PBS) pH 7.2 until $A_{280} < 0.036$. A second wash with 20 mM sodium phosphate, 500 mM NaCl pH 6.2 was performed until $A_{280} < 0.033$. The flow rate was 100 ml/hr and 3 ml fractions were collected. α_2 M was eluted 25 with 100 mM EDTA pH 7.0 at a flow rate of 40 ml/hr. During elution 1 ml fractions were collected.

Recovery of α_2 M was 44%. The α_2 M containing fractions were concentrated to 1 ml on an Amicon devise equipped with a PM 10 membrane and then loaded onto a Superose 12 gelfiltration column (25 mM Tris-HCl, 150 mM NaCl pH 8.0). The α_2 M containing fractions were pooled and stored at -20°C until analysis.

EXAMPLE 3.

Characterization of recombinant human α_2 M.

35

A. Chemical reactions at the thiol ester: thermal fragmentation and methylamine induced cleavage.

A number of different analyses were performed to evaluate the structural and biological characteristics of the human $\alpha_2\text{M}$ as compared to a preparation of human plasma derived $\alpha_2\text{M}$, designated preparation LSJ39.

An important structural feature of $\alpha_2\text{M}$ is the presence of the 5 thiol ester. When heated to 95°C for 15 min, the thiol ester will induce a peptide bond cleavage in the backbone of $\alpha_2\text{M}$ at the position of the thiol esterified Glx-residue. This results in the fragmentation of the 180 kD $\alpha_2\text{M}$ monomer into two polypeptides of 120 kD and 60 kD. Fig. 3 shows an analysis of both the purified $\alpha_2\text{M}$ (from two transformed BHK cell lines) and the 10 purified human plasma derived preparation LSJ39 on a 10-20% SDS polyacrylamide gel. The different preparations, either native human or BHK cell derived recombinant $\alpha_2\text{M}$ were all heat treated to induce thermal fragmentation before loading onto the gel. Molecular weight markers (from top to bottom: 180, 120, 92, 60, 43, 26, 14 and 6 kD) were applied to lanes 1 and 158. Samples in lanes 2, 3 and 4 were not reduced before electrophoresis, while samples in lanes 5, 6 and 7 were reduced. Preparation LSJ39 was applied to lanes 2 and 5. $\alpha_2\text{M}$ K16-6 was applied to lanes 3 and 6, and $\alpha_2\text{M}$ K17-6 was applied to lanes 4 and 7.

It was clear from the patterns of protein fragments on the gel, 20 that both human $\alpha_2\text{M}$ and the two $\alpha_2\text{M}$ preparations showed a considerable degree of thermal fragmentation. As expected, only the reduced samples displayed this fragmentation. In the nonreduced samples, the molecules migrated as the 360 kD dimer.

In the human plasma derived preparation LSJ39 (lane 5) a fragment 25 migrating slightly faster than the 60 kD fragment could be observed. Lanes 6 and 7 indicated the presence in the recombinant material of a similar faster migrating fragment. It is possible that this fragment represented a slightly underglycosylated variant of the 60 kD fragment.

Methylamine (MA) and other small nitrogen containing nucleophilic 30 will cleave the thiol ester and thereby inactivate the ester (Sottrup-Jensen, L., et al., (1980) FEBS Lett. 121: 275-280; Salvesen, G.S. et al., (1981) Biochem. J. 195: 453-461). After MA induced inactivation of the thiol ester, thermal fragmentation of $\alpha_2\text{M}$ can no longer be observed.

Fig. 4 shows a SDS-PAGE run similar to that shown in Fig. 3 (with 35 respect to loaded samples), in which applied $\alpha_2\text{M}$ and $\alpha_2\text{M}$ had been pretreated with MA. From this gel it was concluded, that the thiol ester of $\alpha_2\text{M}$ was just as susceptible to cleavage with MA as the thiol ester of native $\alpha_2\text{M}$. Upon reduction MA-treated $\alpha_2\text{M}$ and $\alpha_2\text{M}$ migrated as a single 180 kD monomer species.

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Lanes 5 of both Fig. 3 and 4 showed an additional band of approximately 85 kD. When α_2 M is cleaved in the bait region by proteinases present in the blood, it generates two fragments, each with a molecular weight of 85 kD. The human α_2 M preparation LSJ39 (purified from serum) 5 contained these cleavage products, while they could not be detected on this gel in the two α_2 M preparations. This indicated that the material secreted from the transformed BHK cell lines was largely native uncomplexed α_2 M. Any α_2 M molecules, that have reacted with proteinases are inactivated and can not form additional complexes with other proteinases. Since the BHK cell 10 does not produce any proteinases that forms complexes with the α_2 M product, this cell is therefore well suited for production of recombinant human α_2 M.

B. Reaction with trypsin.

Reaction with trypsin is a standard way of analyzing the proteinase-complex 15 formation ability of α_2 M (Sottrup-Jensen, L. (1987) in: "The Plasma Proteins" (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Harpel, P.C. (1973) J. Exp. Med. 138: 508-521; Harpel, P.C., et al., (1979) J. Biol. Chem. 254: 8869-8878; Swenson, R.P. and Howard, J.B. (1979) J. Biol. Chem. 254: 4452-4456). In this reaction trypsin will cleave at its target site(s) 20 in the bait region of α_2 M, and the resulting reduced cleavage products (85 kD) will migrate as a double band. Under nonreducing conditions the trypsin- α_2 M complexes will migrate as high molecular weight products.

Fig. 5 shows the result of such an analysis (performed as described (Sottrup-Jensen, L. (1987) in: "The Plasma Proteins" (Putnam, F.W., 25 ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Harpel, P.C. (1973) J. Exp. Med. 138: 508-521; Harpel, P.C., et al., (1979) J. Biol. Chem. 254: 8869-8878; Swenson, R.P. and Howard, J.B. (1979) J. Biol. Chem. 254: 4452-4456)) on the native human α_2 M preparation LSJ39 (lanes 2 and 5) and on α_2 M from cell lines K16-6 (lanes 3 and 6) and K17-6 (lanes 4 and 7). The samples 30 in lanes 2, 3 and 4 were not reduced before electrophoresis, while the samples in lanes 5, 6 and 7 were. Lane 5 shows that almost all of the human native α_2 M was cleaved with trypsin, while the two preparations of α_2 M were cleaved with an efficiency of approximately 80% or more. Without reduction of the complexes no low molecular weight products from the reaction between 35 trypsin and the native α_2 M or the BHK cell derived α_2 M were seen on the gel. The 85 kD fragments derived from the recombinant material migrated somewhat faster than the human standard; as mentioned above the recombinant material might be slightly underglycosylated.

When α_2M is reacted with methylamine, the thiol ester will be inactivated, and α_2M changes conformation from the "slow" form to the "fast" form (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Van Leuven, F., Cassiman, 5 J.-J. and Van Den Berghe, H. (1981) J. Biol. Chem. 256: 9016-9022). In this conformation it can no longer react rapidly with or form complexes with proteinases such as e.g. trypsin.

Fig. 6 shows the results of a set of experiments that were run in parallel to the experiments described above and shown in Fig. 5. However, 10 before reaction with trypsin the native human α_2M and the $r\alpha_2M$ used in this experiment had been treated with methylamine (Sottrup-Jensen, L., et al., (1980) FEBS Lett. 121: 275-280). Under these conditions both the native α_2M and the $r\alpha_2M$ show a marked decrease in reactivity towards trypsin (80% or more of the α_2M and $r\alpha_2M$ monomers were migrating as a 180 kD polypeptide). 15 This indicates that trypsin does not rapidly cleave at the bait region in methylamine treated human α_2M or in BHK cell derived $r\alpha_2M$.

In these types of experiments BHK cell derived $r\alpha_2M$ has shown characteristics similar to those of native human α_2M .

20 C. Trypsin and methylamine induced conformational change in α_2M .

As mentioned above the α_2M molecule will undergo a conformational change both through complex formation with proteinases and through methylamine induced cleavage of the thiol ester. The change in structure results in an altered mobility on rate gels (Sottrup-Jensen, L. (1987) in: The Plasma 25 Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Van Leuven, F., Cassiman, J.-J. and Van Den Berghe, H. (1981) J. Biol. Chem. 256: 9016-9022); unreacted α_2M will migrate as a "slow" form, while reacted α_2M will migrate as a "fast" form.

Fig. 7 and Fig. 8 show these conformational changes, as they 30 appear after reaction with trypsin and methylamine, respectively (analyzed on 5-10% rate gels).

Lanes 1 on both gels contain purified human pregnancy zone protein (PZP) (Sand, O. et al., (1985) J. Biol. Chem. 260: 15723-15735), which is known to appear in both a dimeric (D) and a tetrameric (T) 35 configuration.

Lanes 2 on both gels contain unreacted human α_2M preparation LSJ39. Lanes 3 on both gels show the fast migrating form, resulting from reaction with trypsin and methylamine, respectively. Lanes 4 on both gels show the unreacted $r\alpha_2M$ preparation K16-6, and lanes 5 show the corresponding

fast forms. Lanes 6 on both gels show the unreacted α_2 M preparation K17-6, and lanes 7 show the corresponding fast forms.

It can be concluded that both complex formation between α_2 M and trypsin and reaction of α_2 M with methylamine result in the appearance of 5 fast migrating structures. These structures appear (as analyzed on rate gels) to be very similar to the structures obtained when human α_2 M was allowed to react with trypsin and methylamine. It is also evident from these figures that the α_2 M proteins showed a migration, which, when compared to the migration of dimeric and tetrameric PZP on the gels, is in agreement with the finding that these molecules are produced and secreted from the BHK cells in the active tetrameric conformation.

D. Chromatography of α_2 M on a Superose 6 column.

A Superose 6 column can partially resolve α_2 M molecules in the dimeric configuration from molecules in the tetrameric configuration (Sottrup-Jensen, L. unpublished). Human standard α_2 M and α_2 M was analyzed on a 24 ml Superose 6 column (buffer: 25 mM Tris-HCl, 125 mM NaCl pH 8.0; flow rate: 1 ml/min; fraction size: 1 ml). Fig. 9 shows the diagrams obtained from the chromatography of purified human standard α_2 M and α_2 M from the K17-206 and the K16-6 BHK cell lines. Tetrameric α_2 M (Sottrup-Jensen, unpublished observation) will elute in fraction 12 on this type of column. It is evident from the chromatograms that both of the α_2 M preparations eluted in fraction 12, as did the human standard α_2 M. On this type of column, dimeric α_2 M molecules will elute in fraction 14 and 15 (Sottrup-Jensen, unpublished observation). This type of analysis supported the results obtained from the rate gels (Figs. 7 and 8), that α_2 M was secreted from BHK cells in a tetrameric configuration.

E. Trypsin protection analysis.

30 When trypsin is trapped inside the α_2 M molecule, it retains its catalytic capacity towards low molecular weight substrates such as S-2222 (N-benzoyl-L-Ile-L-Glu-Gly-L-Arg-p-nitroanilide). If trypsin is efficiently complexed with α_2 M, it will be protected against high molecular weight inhibitors such as Soybean Trypsin Inhibitor (STI) (Sottrup-Jensen, L. (1987) 35 in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Ganrot, P.O. (1966) Clin. Chim. Acta. 14: 493-501; Sottrup-Jensen, L. et al., (1981) FEBS Lett. 128: 127-132).

K16-6 and K17-6 derived α_2 M was compared with human plasma α_2 M in such a protection assay. 100 μ l α_2 M (in 25 mM Tris-HCl, 125 mM NaCl, pH

8.0) was mixed with 30 μ l trypsin (0.5 mg/ml in 20 mM sodium acetate pH 5.0). After incubating for 2 min. 30 μ l 1 mg/ml STI (in PBS) was added. 10 μ l aliquots were removed after 2 and 4 min. and each mixed with 750 μ l 0.12 mM S-2222 (dissolved 0.1 M sodiumphosphate pH 8.0, 5% dimethylsulfoxide).

5 The change in absorbance at 405 nm was recorded for 2 min. The results of the assay are given in the following Table II:

TABLE II

10	Prep. of α_2 M.	α_2 M in cuvette.		Activity.
		A_{405}/min	μg	
15	Human LSJ39	0.140	5.00	0.028
	K16-6	0.111	4.62	0.024
	K17-6	0.119	4.87	0.024

20

From these results it can be concluded that α_2 M had essentially the same protection capacity for trypsin against STI as compared with the protection capacity of human plasma α_2 M.

If α_2 M is treated with methylamine before the protection assay, 25 the protection capacity drops dramatically. In a similar assay as that described above, methylamine treated human plasma α_2 M only retained 17% of its protection capacity, while K16-6 and K17-6 α_2 M retained 16% and 14% respectively. It can be concluded that α_2 M protected trypsin against STI with almost the same efficiency as did human plasma α_2 M.

30

E. Amino terminal amino acid sequencing of α_2 M.

Theoretically, the α_2 M characterized in the present investigation could only be either bovine (contaminant from serum), from hamster (endogenous product from the BHK cell) or derived from expression of the 35 transfected plasmid p1167. The ELISA assay used never recognized any α_2 M in BHK cell conditioned medium, whether with or without added fetal calf serum. To make sure that the investigated α_2 M was human α_2 M, and to characterize the amino terminal processing of the recombinant product, amino terminal amino acid sequence determination was carried on out K16-6 and K17-6 α_2 M as 40 described (Sottrup-Jensen, L. et al., (1984) J. Biol. Chem. 259: 8293-8303). The Edman degradation was repeated for 12 cycles, and the identity of the detected amino acid derivative in each cycle, was in total agreement with the

amino terminal sequence of human α_2 M: Ser-Val-Ser-Gly-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val-, whereas bovine α_2 M has the following amino terminal sequence: Ala-Val-Asp-Gly-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val- (unpublished, Dr. Torsten Kristensen, Department of Molecular Biology, University of Aarhus, Denmark.)

5

EXAMPLE 4.

Construction and expression of a bait region mutant of human α_2 M.

In the present example it is demonstrated that the bait region of human α_2 M can be substituted by the bait region of human pregnancy zone protein (PZP) (Sottrup Jensen, L., Folkersen, J., Kristensen, T. and Tack, B.F. Partial primary structure of human pregnancy zone protein: extensive sequence homology with human alpha 2-macroglobulin. Proc. Natl. Acad. Sci. U.S.A. 81, 7353-7357, 1984; Sand, O., Folkersen, J., Westergaard, J.G. and Sottrup Jensen, L. Characterization of human pregnancy zone protein. Comparison with human alpha 2-macroglobulin. J.Biol.Chem. 260, 15723-15735, 1985). The resulting α_2 M bait region mutant exhibited a proteinase inhibitor profile similar to that of human pregnancy zone protein.

To facilitate substitution of DNA fragments encoding the bait region of human α_2 M cDNA, target sites for the restriction enzymes PstI and SacII were introduced at the 5' and at the 3' end of the cDNA region encoding the bait region.

The human α_2 M expression plasmid p1167 was digested with BamHI and ClaI, and a 2660 bp fragment, which carried the central part of the human α_2 M cDNA, was subcloned in the BamHI and ClaI digested vector pSX191.

25 This vector, which had previously been constructed, is a derivative of pUC19. It was constructed as described: pUC19 was digested with EcoRI and HindIII, and a synthetic linker with the following sequence

```

      KpnI  PstI  EcoRI Hind3 ClaI  SphI  BamHI
30 AATTGGTACCCTGCAGGAATTCAAGCTTATCGATGGCATGCGGATCC - NOR781
      CCATGGGACGTCCTTAAGTTCGAATAGCTACCGTACGCCTAGGTCGA - NOR782

```

was cloned in the digested pUC19 vector. The linker, which was an annealing product from the two synthetic oligonucleotides NOR781 and NOR782, has 35 cohesive ends that will ligate to the EcoRI and the HindIII sites of pUC19 in such a way that these ligation sites are not regenerated in the pSX191 vector. Thus pSX191 carried sites for KpnI, PstI, EcoRI, HindIII, ClaI, SphI and BamHI.

The resulting plasmid pSX191 α_2 M was digested with BamHI and HindIII, and a purified 2.6 kb BamHI/HindIII α_2 M fragment was cloned in

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M13mpl8 to generate M13mpl8 α_2 M for mutagenesis by described methods. A synthetic oligonucleotide NOR973, with the following sequence:

5'(TTCATACTGCTGCAGCTGTGGACAC)3'

was used to introduce a PstI site at position 2102 (SEQ ID NO:1) in the cDNA 5 sequence, and a oligonucleotide (NOR974) with the following sequence:

5'(AGCCACCCCGCGGAGTTACCAC)3'

was used to introduce a SacII site at position 2271 (SEQ ID NO:1) in the cDNA sequence. These sites were chosen because they did not introduce alterations in the encoded amino acid sequence, and they were within a 10 convenient distance of the bait region in human α_2 M cDNA. Both primers were used in the same mutagenesis experiment (Kunkel, T.A., Roberts, J.D. and Zakour, R.A. Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection. Methods in Enzymol. 154, 367-382, 1987); dsDNA was isolated from mutated M13mpl8 α_2 M plaques, and the DNA was digested with the restriction 15 enzymes PstI and SacII. Correctly mutated recombinants, which had an insert of 160 bp, were further analyzed by DNA sequencing (Tabor, S. and Richardson, C.C. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 84, 4767-4771, 1987). A 2.6 kb BamHI/HindIII fragment from a correct α_2 M cDNA mutant (M13mpl8 α_2 M#212.1) was subcloned in 20 a BamHI/HindIII digested pUC13 vector, and a correct subclone p1308 was isolated and characterized with BamHI/HindIII and PstI/SacII double digestions and DNA electrophoresis.

The PstI/SacII fragment in p1308 can be excised and replaced with a different DNA fragment, which encodes bait region variants. The 25 resulting new variants (bait region mutants or analogs) of α_2 M cDNA can be isolated as BamHI/ClaI fragments and subcloned back into BamHI/ClaI digested expression vector p1167.

In the present example DNA encoding the amino acids of the bait region for human PZP (Sottrup-Jensen et al. 1989, supra) was obtained from 30 ligation, annealing and cloning of 8 synthetic oligonucleotides.

The DNA sequence of the synthetic fragment and the encoded amino acids as inserted into the α_2 M clone are given in SEQ ID NO:3, and comprises positions 2107 to 2305 and the corresponding amino acids. A PstI site was introduced at the 5' end in the synthetic fragment, and SacII and BamHI sites 35 were introduced at the 3' end.

This synthetic 0.2 kb DNA fragment was cloned in a PstI/BamHI digested M13mpl8 vector for DNA sequencing. DNA from a clone containing the correct sequence was digested with PstI and SacII, and the purified 0.2 kb fragment was cloned in a PstI/SacII digested and gel purified p1308 vector.

A correct recombinant, p267PZP, was characterized with restriction enzyme digestions, and from this plasmid, bait region mutated (α_2 M \rightarrow PZP) cDNA was isolated as a 2.7 kb BamHI/ClaI fragment and subcloned in a BamHI/ClaI digested α_2 M expression vector p1167. The resulting plasmid, designated p1365, was grown as a large scale plasmid preparation, purified by CsCl centrifugation, and cotransfected with pDHFR-I into BHK cells.

Through this procedure the nucleotides 2102 to 2275 in SEQ ID NO:1 was removed and replaced with nucleotides 2102 to 2305 in SEQ ID NO:3.

The procedures for transfection, selection of bait region mutated α_2 M (designated α_2 M-PZP) recombinants (with an α_2 M specific ELISA), large scale production and purification of mutated α_2 M were as described elsewhere (EXAMPLE 2) in this application.

Characterization of the proteinase inhibitor specificity of a bait region mutant of human α_2 M.

The purified recombinant α_2 M mutant, α_2 M-PZP, was characterized with respect to its inhibitor specificity profile against various proteinases by the use of previously described methods (Sand et al.1985). For comparison human plasma derived α_2 M and PZP were treated with the same set of proteinases in parallel reactions. The proteinases used were chymotrypsin, elastase, trypsin and Staphylococcus aureus Glu-specific proteinase. It has been reported (Sand et al.1985) that chymotrypsin and elastase show a rapid reaction with both PZP and α_2 M, while the reaction between the two proteinase inhibitors and trypsin and Staphylococcus aureus Glu-specific proteinase is quite dissimilar for PZP and α_2 M: both proteinases react rapidly with α_2 M, while the reaction with PZP is slow (Sand et al.1985). The reason for this difference in reaction rate with the different proteinases is believed to be due to the fact that the bait region in PZP contains strong specificity determinant for chymotrypsin and elastase, but none for trypsin and Staphylococcus aureus Glu-specific proteinase.

The results of the analysis is presented in figures 10 to 13.

Figure 10 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from chymotrypsin treated human α_2 M, human PZP and α_2 M-PZP. Molecular weight markers (from top to bottom: 180, 120, 92, 35 60, 43, 26, 14 and 6 kD) were applied to lanes 1 and 8. All samples were reduced. Lanes 2, 3 and 4 show the cleavage products obtained from reaction of chymotrypsin with human plasma derived PZP, α_2 M-PZP and human plasma derived α_2 M, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1

between proteinase and the three tested inhibitors. In all 6 lanes cleavage products (85 kD) could be identified. This indicated that α_2 M-PZP reacted with chymotrypsin with similar characteristics as did human plasma derived α_2 M and PZP.

5 Figure 11 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from elastase treated human α_2 M, human PZP and α_2 M-PZP. Molecular weight markers were the same as applied on the gel in Fig. 2. All samples were reduced. Lanes 2, 3 and 4 show the cleavage products obtained from reaction of elastase with human plasma derived PZP, 10 α_2 M-PZP and human plasma derived α_2 M, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1 between proteinase and the three tested inhibitors. In all 6 lanes cleavage products (85 kD) could be identified. This indicated that α_2 M-PZP reacted with elastase with similar characteris-
15 tics as did human plasma derived α_2 M and PZP.

Figure 12 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from trypsin treated human α_2 M, human PZP and α_2 M-PZP. Molecular weight markers were the same as applied on the gel in Fig. 2. All samples were reduced. Lanes 2, 3 and 4 show the cleavage
20 products obtained from reaction of trypsin with human plasma derived PZP, human plasma derived α_2 M and α_2 M-PZP, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1 between proteinase and the three tested inhibitors. In lanes 3 and 6 cleavage products (85 kD) could be identified
25 from the reaction between trypsin and α_2 M. In lanes 2, 4, 5 and 7 no cleavage products were observed from the reaction of trypsin with PZP and α_2 M-PZP. This result demonstrated that α_2 M-PZP reacted poorly with trypsin as did human plasma derived PZP, while α_2 M was cleaved in the reaction with trypsin.

Figure 13 illustrates the gel electrophoresis (10 - 20 % reducing
30 SDS-PAGE) of the reaction products from Staphylococcus aureus Glu-specific protease treated human α_2 M, human PZP and α_2 M-PZP. Molecular weight markers were the same as applied on the gel in Fig. 2. All samples were reduced. Lanes 2, 3 and 4 show the cleavage products obtained from reaction of
35 Staphylococcus aureus Glu-specific protease with human plasma derived PZP, α_2 M-PZP and human plasma derived α_2 M, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1 between proteinase and the three tested inhibitors. In lanes 4 and 7 cleavage products (85 kD) could be identified from the reaction between Staphylococcus aureus Glu-specific protease and

α_2 M. In lanes 2, 3, 5 and 6 much less cleavage product could be identified from the reaction of this proteinase with PZP and $r\alpha_2$ M-PZP. This result demonstrated that $r\alpha_2$ M-PZP reacted poorly with the Staphylococcus aureus proteinase as did human plasma derived PZP, while α_2 M was cleaved in the 5 reaction with this proteinase.

It can be concluded that $r\alpha_2$ M-PZP showed the same pattern of reaction with four proteinases as did human plasma derived PZP. This pattern of reaction was different from the corresponding pattern obtained from reaction with α_2 M. Thus $r\alpha_2$ M-PZP has been demonstrated to have a proteinase 10 inhibitor profile similar to native PZP and dissimilar to α_2 M. Thus it has been demonstrated that the proteinase inhibitor profile of α_2 M can be modulated by substitution of DNA fragments encoding the bait region.

The substitution as described in this invention did not destroy the activity of the proteinase inhibitor, and it is therefore demonstrated 15 that functional macroglobulin hybrids can be constructed by substitutions (mutations) in the bait region. The finding will lead to the design of α_2 M-derivatives with new desired proteinase specificities. No doubt, these results could be extended to other macroglobulin based hybrids, in which the bait region can be modified at will to obtain new inhibitor specificities.

20 Aggressive activity of proteinases is often a problem in relation to various diseases (e.g. the activity of elastase and cathepsin G in severe inflammation leads to tissue and organ destruction and failure). Inhibitors of such proteinases will be useful in drug design. In situations where the target site for the proteinase is known, but no inhibitor can be identified, 25 α_2 M can be engineered (mutated in the bait region) to obtain the desired specificity. In a situation where the target specificity of the proteinase in question is unknown, saturation mutagenesis or random synthesis of the bait region will lead to an indefinite number of target sequences that can be introduced and expressed in hybrid macroglobulins. These hybrids can be 30 screened for proteinase inhibition, and the target sequence(s) can be identified. The resulting α_2 M analog can be produced and purified as described elsewhere in this invention. Upon injection into the circulation such α_2 M analogs will inhibit and clear from the blood any proteinase of the given specificity.

35 Introduction of protein analogs or mutants in the human body always raises the possibility for antigenicity. The generation of a panel of 45 mouse monoclonal antibodies against human α_2 M has been described (Van Leuven et al.1988; Delain et al.1988). None of these antibodies were directed against the bait region. This indicates that the bait region is not highly

antigenic and that mutants in this region of the molecule can be generated and used for therapeutical uses without risk for antibody development.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Novo Nordisk A/S
- (ii) TITLE OF INVENTION: Expression of Plasma Glycoproteins
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Novo Nordisk A/S, Patent Department
 - (B) STREET: Novo Alle
 - (C) CITY: Bagsvaerd
 - (E) COUNTRY: DENMARK
 - (F) ZIP: DK-2880
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: DK 4235/89, DK 4236/89, DK 4237/89
 - (B) FILING DATE: 29-AUG-1989

(2) INFORMATION FOR SEQ ID NO:1:

- ```
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 4569 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

iii) HYPOTHETICAL: N

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
 (F) TISSUE TYPE: Hepatic
 (G) CELL TYPE: Hepatoblastoma
 (H) CELL LINE: HepG2

(ix) FEATURE:
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCTCCTCCA GCTCCTTCTT TCTGCAAC ATG GGG AAG AAC AAA CTC CTT CAT 52  
Met Gly Lys Asn Lys Leu Leu His  
1 5

CCA AGT CTG GTT CTT CTC CTC TTG GTC CTC CTG CCC ACA GAC GCC TCA 100  
Pro Ser Leu Val Leu Leu Leu Leu Val Leu Leu Pro Thr Asp Ala Ser  
10 15 20

35

|                   |            |                   |                  |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |     |
|-------------------|------------|-------------------|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-----|
| GTC<br>Val<br>25  | TCT<br>Ser | GGA<br>Gly        | AAA<br>Lys       | CCG<br>Pro        | CAG<br>Gln<br>30  | TAT<br>Tyr        | ATG<br>Met        | GTT<br>Val        | CTG<br>Leu        | GTC<br>Val<br>35  | CCC<br>Pro        | TCC<br>Ser        | CTG<br>Leu        | CTC<br>Leu        | CAC<br>His<br>40  | 148 |
| ACT<br>Thr        | GAG<br>Glu | ACC<br>Thr        | ACT<br>Thr       | GAG<br>Glu<br>45  | AAG<br>Lys        | GGC<br>Gly        | TGT<br>Cys        | GTC<br>Val        | CTT<br>Leu<br>50  | CTG<br>Leu        | AGC<br>Ser        | TAC<br>Tyr        | CTG<br>Leu        | AAT<br>Asn<br>55  | GAG<br>Glu        | 196 |
| ACA<br>Thr        | GTG<br>Val | ACT<br>Thr        | GTA<br>Val<br>60 | AGT<br>Ser        | GCT<br>Ala        | TCC<br>Ser        | TTG<br>Leu        | GAG<br>Glu<br>65  | TCT<br>Ser        | GTC<br>Val        | AGG<br>Arg        | GGA<br>Gly        | AAC<br>Asn<br>70  | AGG<br>Arg        | AGC<br>Ser        | 244 |
| CTC<br>Leu        | TTC<br>Phe | ACT<br>Thr<br>75  | GAC<br>Asp       | CTG<br>Leu        | GAG<br>Glu        | GCG<br>Ala        | GAG<br>Glu<br>80  | AAT<br>Asn        | GAC<br>Asp        | GTA<br>Val        | CTC<br>Leu        | CAC<br>His<br>85  | TGT<br>Cys        | GTC<br>Val        | GCC<br>Ala        | 292 |
| TTC<br>Phe<br>90  | GCT<br>Ala | GTC<br>Val        | CCA<br>Pro       | AAG<br>Lys        | TCT<br>Ser        | TCA<br>Ser<br>95  | TCC<br>Ser        | AAT<br>Asn        | GAG<br>Glu        | GAG<br>Glu        | GTA<br>Val<br>100 | ATG<br>Met        | TTC<br>Phe        | CTC<br>Leu        | ACT<br>Thr        | 340 |
| GTC<br>Val<br>105 | CAA<br>Gln | GTG<br>Val        | AAA<br>Lys       | GGA<br>Gly        | CCA<br>Pro<br>110 | ACC<br>Thr        | CAA<br>Gln        | GAA<br>Glu        | TTT<br>Phe        | AAG<br>Lys<br>115 | AAG<br>Lys        | CGG<br>Arg        | ACC<br>Thr        | ACA<br>Thr        | GTG<br>Val<br>120 | 388 |
| ATG<br>Met        | GTT<br>Val | AAG<br>Lys        | AAC<br>Asn       | GAG<br>Glu<br>125 | GAC<br>Asp        | AGT<br>Ser        | CTG<br>Leu        | GTC<br>Val        | TTT<br>Phe<br>130 | GTC<br>Val        | CAG<br>Gln        | ACA<br>Thr        | GAC<br>Asp        | AAA<br>Lys<br>135 | TCA<br>Ser        | 436 |
| ATC<br>Ile        | TAC<br>Tyr | AAA<br>Lys<br>140 | CCA<br>Pro       | GGG<br>Gly        | CAG<br>Gln        | ACA<br>Thr        | GTG<br>Val        | AAA<br>Lys<br>145 | TTT<br>Phe        | CGT<br>Arg        | GTT<br>Val        | GTC<br>Val        | TCC<br>Ser<br>150 | ATG<br>Met        | GAT<br>Asp        | 484 |
| GAA<br>Glu        | AAC<br>Asn | TTT<br>Phe<br>155 | CAC<br>His       | CCC<br>Pro        | CTG<br>Leu        | AAT<br>Asn        | GAG<br>Glu<br>160 | TTG<br>Leu        | ATT<br>Ile        | CCA<br>Pro        | CTA<br>Leu        | GTA<br>Val<br>165 | TAC<br>Tyr        | ATT<br>Ile        | CAG<br>Gln        | 532 |
| GAT<br>Asp<br>170 | CCC<br>Pro | AAA<br>Lys        | GGA<br>Gly       | AAT<br>Asn        | CGC<br>Arg        | ATC<br>Ile<br>175 | GCA<br>Ala        | CAA<br>Gln        | TGG<br>Trp        | CAG<br>Gln        | AGT<br>Ser<br>180 | TTC<br>Phe        | CAG<br>Gln        | TTA<br>Leu        | GAG<br>Glu        | 580 |
| GGT<br>Gly<br>185 | GGC<br>Gly | CTC<br>Leu        | AAG<br>Lys       | CAA<br>Gln<br>190 | TTT<br>Phe        | TCT<br>Ser        | TTT<br>Phe        | CCC<br>Pro        | CTC<br>Leu        | TCA<br>Ser<br>195 | TCA<br>Ser        | GAG<br>Glu        | CCC<br>Pro        | TTC<br>Phe        | CAG<br>Gln<br>200 | 628 |
| GGC<br>Gly        | TCC<br>Ser | TAC<br>Tyr        | AAG<br>Lys       | GTG<br>Val<br>205 | GTG<br>Val        | GTA<br>Val        | CAG<br>Gln        | AAG<br>Lys        | AAA<br>Lys<br>210 | TCA<br>Ser        | GGT<br>Gly        | GGA<br>Gly        | AGG<br>Arg        | ACA<br>Thr        | GAG<br>Glu        | 676 |
| CAC<br>His        | CCT<br>Pro | TTC<br>Phe<br>220 | ACC<br>Thr       | GTG<br>Val        | GAG<br>Glu        | GAA<br>Glu        | TTT<br>Phe        | GTT<br>Val<br>225 | CTT<br>Leu        | CCC<br>Pro        | AAG<br>Lys        | TTT<br>Phe        | GAA<br>Glu<br>230 | GTA<br>Val        | CAA<br>Gln        | 724 |
| GTA<br>Val        | ACA<br>Thr | GTG<br>Val<br>235 | CCA<br>Pro       | AAG<br>Lys        | ATA<br>Ile        | ATC<br>Ile        | ACC<br>Thr<br>240 | ATC<br>Ile        | TTG<br>Leu        | GAA<br>Glu        | GAA<br>Glu        | GAG<br>Glu<br>245 | ATG<br>Met        | AAT<br>Asn        | GTA<br>Val        | 772 |

36

|                                                                                                                                                       |      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| TCA GTG TGT GGC CTA TAC ACA TAT GGG AAG CCT GTC CCT GGA CAT GTG<br>Ser Val Cys Gly Leu Tyr Thr Tyr Gly Lys Pro Val Pro Gly His Val<br>250 255 260     | 820  |
| ACT GTG AGC ATT TGC AGA AAG TAT AGT GAC GCT TCC GAC TGC CAC GGT<br>Thr Val Ser Ile Cys Arg Lys Tyr Ser Asp Ala Ser Asp Cys His Gly<br>265 270 275 280 | 868  |
| GAA GAT TCA CAG GCT TTC TGT GAG AAA TTC AGT GGA CAG CTA AAC AGC<br>Glu Asp Ser Gln Ala Phe Cys Glu Lys Phe Ser Gly Gln Leu Asn Ser<br>285 290 295     | 916  |
| CAT GGC TGC TTC TAT CAG CAA GTA AAA ACC AAG GTC TTC CAG CTG AAG<br>His Gly Cys Phe Tyr Gln Gln Val Lys Thr Lys Val Phe Gln Leu Lys<br>300 305 310     | 964  |
| AGG AAG GAG TAT GAA ATG AAA CTT CAC ACT GAG GCC CAG ATC CAA GAA<br>Arg Lys Glu Tyr Glu Met Lys Leu His Thr Glu Ala Gln Ile Gln Glu<br>315 320 325     | 1012 |
| GAA GGA ACA GTG GTG GAA TTG ACT GGA AGG CAG TCC AGT GAA ATC ACA<br>Glu Gly Thr Val Val Glu Leu Thr Gly Arg Gln Ser Ser Glu Ile Thr<br>330 335 340     | 1060 |
| AGA ACC ATA ACC AAA CTC TCA TTT GTG AAA GTG GAC TCA CAC TTT CGA<br>Arg Thr Ile Thr Lys Leu Ser Phe Val Lys Val Asp Ser His Phe Arg<br>345 350 355 360 | 1108 |
| CAG GGA ATT CCC TTC TTT GGG CAG GTG CGC CTA GTA GAT GGG AAA GGC<br>Gln Gly Ile Pro Phe Phe Gly Gln Val Arg Leu Val Asp Gly Lys Gly<br>365 370 375     | 1156 |
| GTC CCT ATA CCA AAT AAA GTC ATA TTC ATC AGA GGA AAT GAA GCA AAC<br>Val Pro Ile Pro Asn Lys Val Ile Phe Ile Arg Gly Asn Glu Ala Asn<br>380 385 390     | 1204 |
| TAT TAC TCC AAT GCT ACC ACG GAT GAG CAT GGC CTT GTA CAG TTC TCT<br>Tyr Tyr Ser Asn Ala Thr Thr Asp Glu His Gly Leu Val Gln Phe Ser<br>395 400 405     | 1252 |
| ATC AAC ACC ACC AAT GTT ATG GGT ACC TCT CTT ACT GTT AGG GTC AAT<br>Ile Asn Thr Thr Asn Val Met Gly Thr Ser Leu Thr Val Arg Val Asn<br>410 415 420     | 1300 |
| TAC AAG GAT CGT AGT CCC TGT TAC GGC TAC CAG TGG GTG TCA GAA GAA<br>Tyr Lys Asp Arg Ser Pro Cys Tyr Gly Tyr Gln Trp Val Ser Glu Glu<br>425 430 435 440 | 1348 |
| CAC GAA GAG GCA CAT CAC ACT GCT TAT CTT GTG TTC TCC CCA AGC AAG<br>His Glu Glu Ala His His Thr Ala Tyr Leu Val Phe Ser Pro Ser Lys<br>445 450 455     | 1396 |
| AGC TTT GTC CAC CTT GAG CCC ATG TCT CAT GAA CTA CCC TGT GGC CAT<br>Ser Phe Val His Leu Glu Pro Met Ser His Glu Leu Pro Cys Gly His<br>460 465 470     | 1444 |

|                                                                                                                                                       |      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| ACT CAG ACA GTC CAG GCA CAT TAT ATT CTG AAT GGA GGC ACC CTG CTG<br>Thr Gln Thr Val Gln Ala His Tyr Ile Leu Asn Gly Gly Thr Leu Leu<br>475 480 485     | 1492 |
| GGG CTG AAG AAG CTC TCC TTC TAT TAT CTG ATA ATG GCA AAG GGA GGC<br>Gly Leu Lys Lys Leu Ser Phe Tyr Tyr Leu Ile Met Ala Lys Gly Gly<br>490 495 500     | 1540 |
| ATT GTC CGA ACT GGG ACT CAT GGA CTG CTT GTG AAG CAG GAA GAC ATG<br>Ile Val Arg Thr Gly Thr His Gly Leu Leu Val Lys Gln Glu Asp Met<br>505 510 515 520 | 1588 |
| AAG GGC CAT TTT TCC ATC TCA ATC CCT GTG AAG TCA GAC ATT GCT CCT<br>Lys Gly His Phe Ser Ile Ser Ile Pro Val Lys Ser Asp Ile Ala Pro<br>525 530 535     | 1636 |
| GTC GCT CGG TTG CTC ATC TAT GCT GTT TTA CCT ACC GGG GAC GTG ATT<br>Val Ala Arg Leu Leu Ile Tyr Ala Val Leu Pro Thr Gly Asp Val Ile<br>540 545 550     | 1684 |
| GGG GAT TCT GCA AAA TAT GAT GTT GAA AAT TGT CTG GCC AAC AAG GTG<br>Gly Asp Ser Ala Lys Tyr Asp Val Glu Asn Cys Leu Ala Asn Lys Val<br>555 560 565     | 1732 |
| GAT TTG AGC TTC AGC CCA TCA CAA AGT CTC CCA GCC TCA CAC GCC CAC<br>Asp Leu Ser Phe Ser Pro Ser Gln Ser Leu Pro Ala Ser His Ala His<br>570 575 580     | 1780 |
| CTG CGA GTC ACA GCG GCT CCT CAG TCC GTC TGC GCC CTC CGT GCT GTG<br>Leu Arg Val Thr Ala Ala Pro Gln Ser Val Cys Ala Leu Arg Ala Val<br>585 590 595 600 | 1828 |
| GAC CAA AGC GTG CTG CTC ATG AAG CCT GAT GCT GAG CTC TCG GCG TCC<br>Asp Gln Ser Val Leu Leu Met Lys Pro Asp Ala Glu Leu Ser Ala Ser<br>605 610 615     | 1876 |
| TCG GTT TAC ACC CTG CTA CCA GAA AAG GAC CTC ACT GGC TTC CCT GGG<br>Ser Val Tyr Asn Leu Leu Pro Glu Lys Asp Leu Thr Gly Phe Pro Gly<br>620 625 630     | 1924 |
| CCT TTG AAT GAC CAG GAC GAT GAA GAC TGC ATC AAT CGT CAT AAT GTC<br>Pro Leu Asn Asp Gln Asp Asp Glu Asp Cys Ile Asn Arg His Asn Val<br>635 640 645     | 1972 |
| TAT ATT AAT GGA ATC ACA TAT ACT CCA GTA TCA AGT ACA AAT GAA AAG<br>Tyr Ile Asn Gly Ile Thr Tyr Thr Pro Val Ser Ser Thr Asn Glu Lys<br>650 655 660     | 2020 |
| GAT ATG TAC AGC TTC CTA GAG GAC ATG GGC TTA AAG GCA TTC ACC AAC<br>Asp Met Tyr Ser Phe Leu Glu Asp Met Gly Leu Lys Ala Phe Thr Asn<br>665 670 675 680 | 2068 |
| TCA AAG ATT CGT AAA CCC AAA ATG TGT CCA CAG CTT CAA CAG TAT GAA<br>Ser Lys Ile Arg Lys Pro Lys Met Cys Pro Gln Leu Gln Gln Tyr Glu<br>685 690 695     | 2116 |

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|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| ATG CAT GGA CCT GAA GGT CTA CGT GTA GGT TTT TAT GAG TCA GAT GTA<br>Met His Gly Pro Glu Gly Leu Arg Val Gly Phe Tyr Glu Ser Asp Val<br>700 705 710     | 2164 |
| ATG GGA AGA GGC CAT GCA CGC CTG GTG CAT GTT GAA GAG CCT CAC ACG<br>Met Gly Arg Gly His Ala Arg Leu Val His Val Glu Glu Pro His Thr<br>715 720 725     | 2212 |
| GAG ACC GTA CGA AAG TAC TTC CCT GAG ACA TGG ATC TGG GAT TTG GTG<br>Glu Thr Val Arg Lys Tyr Phe Pro Glu Thr Trp Ile Trp Asp Leu Val<br>730 735 740     | 2260 |
| GTG GTA AAC TCA GCA GGT GTG GCT GAG GTA GGA GTA ACA GTC CCT GAC<br>Val Val Asn Ser Ala Gly Val Ala Glu Val Gly Val Thr Val Pro Asp<br>745 750 755 760 | 2308 |
| ACC ATC ACC GAG TGG AAG GCA GGG GCC TTC TGC CTG TCT GAA GAT GCT<br>Thr Ile Thr Glu Trp Lys Ala Gly Ala Phe Cys Leu Ser Glu Asp Ala<br>765 770 775     | 2356 |
| GGA CTT GGT ATC TCT TCC ACT GCC TCT CTC CGA GCC TTC CAG CCC TTC<br>Gly Leu Gly Ile Ser Ser Thr Ala Ser Leu Arg Ala Phe Gln Pro Phe<br>780 785 790     | 2404 |
| TTT GTG GAG CTT ACA ATG CCT TAC TCT GTG ATT CGT GGA GAG GCC TTC<br>Phe Val Glu Leu Thr Met Pro Tyr Ser Val Ile Arg Gly Glu Ala Phe<br>795 800 805     | 2452 |
| ACA CTC AAG GCC ACG GTC CTA AAC TAC CTT CCC AAA TGC ATC CGG GTC<br>Thr Leu Lys Ala Thr Val Leu Asn Tyr Leu Pro Lys Cys Ile Arg Val<br>810 815 820     | 2500 |
| AGT GTG CAG CTG GAA GCC TCT CCC GCC TTC CTA GCT GTC CCA GTG GAG<br>Ser Val Gln Leu Glu Ala Ser Pro Ala Phe Leu Ala Val Pro Val Glu<br>825 830 835 840 | 2548 |
| AAG GAA CAA GCG CCT CAC TGC ATC TGT GCA AAC GGG CGG CAA ACT GTG<br>Lys Glu Gln Ala Pro His Cys Ile Cys Ala Asn Gly Arg Gln Thr Val<br>845 850 855     | 2596 |
| TCC TGG GCA GTA ACC CCA AAG TCA TTA GGA AAT GTG AAT TTC ACT GTG<br>Ser Trp Ala Val Thr Pro Lys Ser Leu Gly Asn Val Asn Phe Thr Val<br>860 865 870     | 2644 |
| AGC GCA GAG GCA CTA GAG TCT CAA GAG CTG TGT GGG ACT GAG GTG CCT<br>Ser Ala Glu Ala Leu Glu Ser Gln Glu Leu Cys Gly Thr Glu Val Pro<br>875 880 885     | 2692 |
| TCA GTT CCT GAA CAC GGA AGG AAA GAC ACA GTC ATC AAG CCT CTG TTG<br>Ser Val Pro Glu His Gly Arg Lys Asp Thr Val Ile Lys Pro Leu Leu<br>890 895 900     | 2740 |
| GTT GAA CCT GAA GGA CTA GAG AAG GAA ACA ACA TTC AAC TCC CTA CTT<br>Val Glu Pro Glu Gly Leu Glu Lys Glu Thr Thr Phe Asn Ser Leu Leu<br>905 910 915 920 | 2788 |



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|------------------------------------------------------------------------------------------------------------------------------------|------|
| TGT CCA TCA GGT GGT GAG GTT TCT GAA GAA TTA TCC CTG AAA CTG CCA<br>Cys Pro Ser Gly Gly Glu Val Ser Glu Glu Leu Ser Leu Lys Leu Pro | 2836 |
| 925 930 935                                                                                                                        |      |
| CCA AAT GTG GTA GAA GAA TCT GCC CGA GCT TCT GTC TCA GTT TTG GGA<br>Pro Asn Val Val Glu Glu Ser Ala Arg Ala Ser Val Ser Val Leu Gly | 2884 |
| 940 945 950                                                                                                                        |      |
| GAC ATA TTA GGC TCT GCC ATG CAA AAC ACA CAA AAT CTT CTC CAG ATG<br>Asp Ile Leu Gly Ser Ala Met Gln Asn Thr Gln Asn Leu Leu Gln Met | 2932 |
| 955 960 965                                                                                                                        |      |
| CCC TAT GGC TGT GGA GAG CAG AAT ATG GTC CTC TTT GCT CCT AAC ATC<br>Pro Tyr Gly Cys Gly Glu Gln Asn Met Val Leu Phe Ala Pro Asn Ile | 2980 |
| 970 975 980                                                                                                                        |      |
| TAT GTA CTG GAT TAT CTA AAT GAA ACA CAG CAG CTT ACT CCA GAG ATC<br>Tyr Val Leu Asp Tyr Leu Asn Glu Thr Gln Gln Leu Thr Pro Glu Ile | 3028 |
| 985 990 995 1000                                                                                                                   |      |
| AAG TCC AAG GCC ATT GGC TAT CTC AAC ACT GGT TAC CAG AGA CAG TTG<br>Lys Ser Lys Ala Ile Gly Tyr Leu Asn Thr Gly Tyr Gln Arg Gln Leu | 3076 |
| 1005 1010 1015                                                                                                                     |      |
| AAC TAC AAA CAC TAT GAT GGC TCC TAC AGC ACC TTT GGG GAG CGA TAT<br>Asn Tyr Lys His Tyr Asp Gly Ser Tyr Ser Thr Phe Gly Glu Arg Tyr | 3124 |
| 1020 1025 1030                                                                                                                     |      |
| GGC AGG AAC CAG GGC AAC ACC TGG CTC ACA GCC TTT GTT CTG AAG ACT<br>Gly Arg Asn Gln Gly Asn Thr Trp Leu Thr Ala Phe Val Leu Lys Thr | 3172 |
| 1035 1040 1045                                                                                                                     |      |
| TTT GCC CAA GCT CGA GCC TAC ATC TTC ATC GAT GAA GCA CAC ATT ACC<br>Phe Ala Gln Ala Arg Ala Tyr Ile Phe Ile Asp Glu Ala His Ile Thr | 3220 |
| 1050 1055 1060                                                                                                                     |      |
| CAA GCC CTC ATA TGG CTC TCC CAG AGG CAG AAG GAC AAT GGC TGT TTC<br>Gln Ala Leu Ile Trp Leu Ser Gln Arg Gln Lys Asp Asn Gly Cys Phe | 3268 |
| 1065 1070 1075 1080                                                                                                                |      |
| AGG AGC TCT GGG TCA CTG CTC AAC AAT GCC ATA AAG GGA GGA GTA GAA<br>Arg Ser Ser Gly Ser Leu Leu Asn Asn Ala Ile Lys Gly Gly Val Glu | 3316 |
| 1085 1090 1095                                                                                                                     |      |
| GAT GAA GTG ACC CTC TCC GCC TAT ATC ACC ATC GCC CTT CTG GAG ATT<br>Asp Glu Val Thr Leu Ser Ala Tyr Ile Thr Ile Ala Leu Leu Glu Ile | 3364 |
| 1100 1105 1110                                                                                                                     |      |
| CCT CTC ACA GTC ACT CAC CCT GTT GTC CGC AAT GCC CTG TTT TGC CTG<br>Pro Leu Thr Val Thr His Pro Val Val Arg Asn Ala Leu Phe Cys Leu | 3412 |
| 1115 1120 1125                                                                                                                     |      |
| GAG TCA GCC TGG AAG ACA GCA CAA GAA GGG GAC CAT GGC AGC CAT GTA<br>Glu Ser Ala Trp Lys Thr Ala Gln Glu Gly Asp His Gly Ser His Val | 3460 |
| 1130 1135 1140                                                                                                                     |      |

40

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| TAT ACC AAA GCA CTG CTG GCC TAT GCT TTT GCC CTG GCA GGT AAC CAG<br>Tyr Thr Lys Ala Leu Leu Ala Tyr Ala Phe Ala Leu Ala Gly Asn Gln<br>1145 1150 1155 1160 | 3508 |
| GAC AAG AGG AAG GAA GTA CTC AAG TCA CTT AAT GAG GAA GCT GTG AAG<br>Asp Lys Arg Lys Glu Val Leu Lys Ser Leu Asn Glu Glu Ala Val Lys<br>1165 1170 1175      | 3556 |
| AAA GAC AAC TCT GTC CAT TGG GAG CGC CCT CAG AAA CCC AAG GCA CCA<br>Lys Asp Asn Ser Val His Trp Glu Arg Pro Gln Lys Pro Lys Ala Pro<br>1180 1185 1190      | 3604 |
| GTG GGG CAT TTT TAC GAA CCC CAG GCT CCC TCT GCT GAG GTG GAG ATG<br>Val Gly His Phe Tyr Glu Pro Gln Ala Pro Ser Ala Glu Val Glu Met<br>1195 1200 1205      | 3652 |
| ACA TCC TAT GTG CTC CTC GCT TAT CTC ACG GCC CAG CCA GCC CCA ACC<br>Thr Ser Tyr Val Leu Leu Ala Tyr Leu Thr Ala Gln Pro Ala Pro Thr<br>1210 1215 1220      | 3700 |
| TCG GAG GAC CTG ACC TCT GCA ACC AAC ATC GTG AAG TGG ATC ACG AAG<br>Ser Glu Asp Leu Thr Ser Ala Thr Asn Ile Val Lys Trp Ile Thr Lys<br>1225 1230 1235 1240 | 3748 |
| CAG CAG AAT GCC CAG GGC GGT TTC TCC TCC ACC CAG CAC ACA GTG GTG<br>Gln Gln Asn Ala Gln Gly Gly Phe Ser Ser Thr Gln His Thr Val Val<br>1245 1250 1255      | 3796 |
| GCT CTC CAT GCT CTG TCC AAA TAT GGA GCA GCC ACA TTT ACC AGG ACT<br>Ala Leu His Ala Leu Ser Lys Tyr Gly Ala Ala Thr Phe Thr Arg Thr<br>1260 1265 1270      | 3844 |
| GGG AAG GCT GCA CAG GTG ACT ATC CAG TCT TCA GGG ACA TTT TCC AGC<br>Gly Lys Ala Ala Gln Val Thr Ile Gln Ser Ser Gly Thr Phe Ser Ser<br>1275 1280 1285      | 3892 |
| AAA TTC CAA GTG GAC AAC AAC AAC CGC CTG TTA CTG CAG CAG GTC TCA<br>Lys Phe Gln Val Asp Asn Asn Asn Arg Leu Leu Leu Gln Gln Val Ser<br>1290 1295 1300      | 3940 |
| TTG CCA GAG CTG CCT GGG GAA TAC AGC ATG AAA GTG ACA GGA GAA GGA<br>Leu Pro Glu Leu Pro Gly Glu Tyr Ser Met Lys Val Thr Gly Glu Gly<br>1305 1310 1315 1320 | 3988 |
| TGT GTC TAC CTC CAG ACA TCC TTG AAA TAC AAT ATT CTC CCA GAA AAG<br>Cys Val Tyr Leu Gln Thr Ser Leu Lys Tyr Asn Ile Leu Pro Glu Lys<br>1325 1330 1335      | 4036 |
| GAA GAG TTC CCC TTT GCT TTA GGA GTG CAG ACT CTG CCT CAA ACT TGT<br>Glu Glu Phe Pro Phe Ala Leu Gly Val Gln Thr Leu Pro Gln Thr Cys<br>1340 1345 1350      | 4084 |
| GAT GAA CCC AAA GCC CAC ACC AGC TTC CAA ATC TCC CTA AGT GTC AGT<br>Asp Glu Pro Lys Ala His Thr Ser Phe Gln Ile Ser Leu Ser Val Ser<br>1355 1360 1365      | 4132 |

41

TAC ACA GGG AGC CGC TCT GCC TCC AAC ATG GCG ATC GTT GAT GTG AAG 4180  
 Tyr Thr Gly Ser Arg Ser Ala Ser Asn Met Ala Ile Val Asp Val Lys  
 1370 1375 1380

ATG GTC TCT GGC TTC ATT CCC CTG AAG CCA ACA GTG AAA ATG CTT GAA 4228  
 Met Val Ser Gly Phe Ile Pro Leu Lys Pro Thr Val Lys Met Leu Glu  
 1385 1390 1395 1400

AGA TCT AAC CAT GTG AGC CGG ACA GAA GTC AGC AGC AAC CAT GTC TTG 4276  
 Arg Ser Asn His Val Ser Arg Thr Glu Val Ser Ser Asn His Val Leu  
 1405 1410 1415

ATT TAC CTT GAT AAG GTG TCA AAT CAG ACA CTG AGC TTG TTC TTC ACG 4324  
 Ile Tyr Leu Asp Lys Val Ser Asn Gln Thr Leu Ser Leu Phe Phe Thr  
 1420 1425 1430

GTT CTG CAA GAT GTC CCA GTA AGA GAT CTC AAA CCA GCC ATA GTG AAA 4372  
 Val Leu Gln Asp Val Pro Val Arg Asp Leu Lys Pro Ala Ile Val Lys  
 1435 1440 1445

GTC TAT GAT TAC TAC GAG ACG GAT GAG TTT GCA ATT GCT GAG TAC AAT 4420  
 Val Tyr Asp Tyr Tyr Glu Thr Asp Glu Phe Ala Ile Ala Glu Tyr Asn  
 1450 1455 1460

GCT CCT TGC AGC AAA GAT CTT GGA AAT GCT TGAAGACCAC AAGGCTGAAA 4470  
 Ala Pro Cys Ser Lys Asp Leu Gly Asn Ala  
 1465 1470

AGTGCTTTGC TGGAGTCCTG TTCTCTGAGC TCCACAGAAG ACACGTGTTT TTGTATCTTT 4530  
 AAAGACTTGA TGAATAAACA CTTTTTCTGG TCAAAAAAA 4569

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1474 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
  - (E) FEATURES: bait region: 690-730
- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Lys Asn Lys Leu Leu His Pro Ser Leu Val Leu Leu Leu Leu  
 1 5 10 15

Val Leu Leu Pro Thr Asp Ala Ser Val Ser Gly Lys Pro Gln Tyr Met  
 20 25 30

Val Leu Val Pro Ser Leu Leu His Thr Glu Thr Thr Glu Lys Gly Cys  
 35 40 45

Val Leu Leu Ser Tyr Leu Asn Glu Thr Val Thr Val Ser Ala Ser Leu  
 50 55 60

Glu Ser Val Arg Gly Asn Arg Ser Leu Phe Thr Asp Leu Glu Ala Glu  
 65 70 75 80

|            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |
|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Asn        | Asp        | Val        | Leu        | His<br>85  | Cys        | Val        | Ala        | Phe        | Ala<br>90  | Val        | Pro        | Lys        | Ser        | Ser<br>95  | Ser        |
| Asn        | Glu        | Glu        | Val<br>100 | Met        | Phe        | Leu        | Thr        | Val<br>105 | Gln        | Val        | Lys        | Gly        | Pro<br>110 | Thr        | Gln        |
| Glu        | Phe        | Lys<br>115 | Lys        | Arg        | Thr        | Thr        | Val<br>120 | Met        | Val        | Lys        | Asn        | Glu<br>125 | Asp        | Ser        | Leu        |
| Val        | Phe<br>130 | Val        | Gln        | Thr        | Asp        | Lys<br>135 | Ser        | Ile        | Tyr        | Lys        | Pro<br>140 | Gly        | Gln        | Thr        | Val        |
| Lys<br>145 | Phe        | Arg        | Val        | Val        | Ser<br>150 | Met        | Asp        | Glu        | Asn        | Phe<br>155 | His        | Pro        | Leu        | Asn        | Glu<br>160 |
| Leu        | Ile        | Pro        | Leu        | Val<br>165 | Tyr        | Ile        | Gln        | Asp        | Pro<br>170 | Lys        | Gly        | Asn        | Arg        | Ile<br>175 | Ala        |
| Gln        | Trp        | Gln        | Ser<br>180 | Phe        | Gln        | Leu        | Glu        | Gly<br>185 | Gly        | Leu        | Lys        | Gln        | Phe<br>190 | Ser        | Phe        |
| Pro        | Leu        | Ser<br>195 | Ser        | Glu        | Pro        | Phe        | Gln<br>200 | Gly        | Ser        | Tyr        | Lys        | Val<br>205 | Val        | Val        | Gln        |
| Lys        | Lys<br>210 | Ser        | Gly        | Gly        | Arg        | Thr<br>215 | Glu        | His        | Pro        | Phe        | Thr<br>220 | Val        | Glu        | Glu        | Phe        |
| Val<br>225 | Leu        | Pro        | Lys        | Phe        | Glu<br>230 | Val        | Gln        | Val        | Thr        | Val<br>235 | Pro        | Lys        | Ile        | Ile        | Thr<br>240 |
| Ile        | Leu        | Glu        | Glu        | Glu<br>245 | Met        | Asn        | Val        | Ser        | Val<br>250 | Cys        | Gly        | Leu        | Tyr        | Thr<br>255 | Tyr        |
| Gly        | Lys        | Pro        | Val<br>260 | Pro        | Gly        | His        | Val        | Thr<br>265 | Val        | Ser        | Ile        | Cys        | Arg<br>270 | Lys        | Tyr        |
| Ser        | Asp        | Ala<br>275 | Ser        | Asp        | Cys        | His        | Gly<br>280 | Glu        | Asp        | Ser        | Gln        | Ala<br>285 | Phe        | Cys        | Glu        |
| Lys        | Phe<br>290 | Ser        | Gly        | Gln        | Leu        | Asn<br>295 | Ser        | His        | Gly        | Cys        | Phe<br>300 | Tyr        | Gln        | Gln        | Val        |
| Lys<br>305 | Thr        | Lys        | Val        | Phe        | Gln<br>310 | Leu        | Lys        | Arg        | Lys        | Glu<br>315 | Tyr        | Glu        | Met        | Lys        | Leu<br>320 |
| His        | Thr        | Glu        | Ala        | Gln<br>325 | Ile        | Gln        | Glu        | Glu        | Gly<br>330 | Thr        | Val        | Val        | Glu        | Leu<br>335 | Thr        |
| Gly        | Arg        | Gln        | Ser<br>340 | Ser        | Glu        | Ile        | Thr        | Arg<br>345 | Thr        | Ile        | Thr        | Lys        | Leu<br>350 | Ser        | Phe        |
| Val        | Lys<br>355 | Val        | Asp        | Ser        | His        | Phe        | Arg<br>360 | Gln        | Gly        | Ile        | Pro        | Phe<br>365 | Phe        | Gly        | Gln        |
| Val        | Arg<br>370 | Leu        | Val        | Asp        | Gly        | Lys<br>375 | Gly        | Val        | Pro        | Ile        | Pro<br>380 | Asn        | Lys        | Val        | Ile        |

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Phe Ile Arg Gly Asn Glu Ala Asn Tyr Tyr Ser Asn Ala Thr Thr Asp  
 385 390 395 400  
 Glu His Gly Leu Val Gln Phe Ser Ile Asn Thr Thr Asn Val Met Gly  
 405 410 415  
 Thr Ser Leu Thr Val Arg Val Asn Tyr Lys Asp Arg Ser Pro Cys Tyr  
 420 425 430  
 Gly Tyr Gln Trp Val Ser Glu Glu His Glu Glu Ala His His Thr Ala  
 435 440 445  
 Tyr Leu Val Phe Ser Pro Ser Lys Ser Phe Val His Leu Glu Pro Met  
 450 455 460  
 Ser His Glu Leu Pro Cys Gly His Thr Gln Thr Val Gln Ala His Tyr  
 465 470 475 480  
 Ile Leu Asn Gly Gly Thr Leu Leu Gly Leu Lys Lys Leu Ser Phe Tyr  
 485 490 495  
 Tyr Leu Ile Met Ala Lys Gly Gly Ile Val Arg Thr Gly Thr His Gly  
 500 505 510  
 Leu Leu Val Lys Gln Glu Asp Met Lys Gly His Phe Ser Ile Ser Ile  
 515 520 525  
 Pro Val Lys Ser Asp Ile Ala Pro Val Ala Arg Leu Leu Ile Tyr Ala  
 530 535 540  
 Val Leu Pro Thr Gly Asp Val Ile Gly Asp Ser Ala Lys Tyr Asp Val  
 545 550 555 560  
 Glu Asn Cys Leu Ala Asn Lys Val Asp Leu Ser Phe Ser Pro Ser Gln  
 565 570 575  
 Ser Leu Pro Ala Ser His Ala His Leu Arg Val Thr Ala Ala Pro Gln  
 580 585 590  
 Ser Val Cys Ala Leu Arg Ala Val Asp Gln Ser Val Leu Leu Met Lys  
 595 600 605  
 Pro Asp Ala Glu Leu Ser Ala Ser Ser Val Tyr Asn Leu Leu Pro Glu  
 610 615 620  
 Lys Asp Leu Thr Gly Phe Pro Gly Pro Leu Asn Asp Gln Asp Asp Glu  
 625 630 635 640  
 Asp Cys Ile Asn Arg His Asn Val Tyr Ile Asn Gly Ile Thr Tyr Thr  
 645 650 655  
 Pro Val Ser Ser Thr Asn Glu Lys Asp Met Tyr Ser Phe Leu Glu Asp  
 660 665 670  
 Met Gly Leu Lys Ala Phe Thr Asn Ser Lys Ile Arg Lys Pro Lys Met  
 675 680 685

44

Cys Pro Gln Leu Gln Gln Tyr Glu Met His Gly Pro Glu Gly Leu Arg  
 690 695 700  
 Val Gly Phe Tyr Glu Ser Asp Val Met Gly Arg Gly His Ala Arg Leu  
 705 710 715 720  
 Val His Val Glu Glu Pro His Thr Glu Thr Val Arg Lys Tyr Phe Pro  
 725 730 735  
 Glu Thr Trp Ile Trp Asp Leu Val Val Val Asn Ser Ala Gly Val Ala  
 740 745 750  
 Glu Val Gly Val Thr Val Pro Asp Thr Ile Thr Glu Trp Lys Ala Gly  
 755 760 765  
 Ala Phe Cys Leu Ser Glu Asp Ala Gly Leu Gly Ile Ser Ser Thr Ala  
 770 775 780  
 Ser Leu Arg Ala Phe Gln Pro Phe Phe Val Glu Leu Thr Met Pro Tyr  
 785 790 795 800  
 Ser Val Ile Arg Gly Glu Ala Phe Thr Leu Lys Ala Thr Val Leu Asn  
 805 810 815  
 Tyr Leu Pro Lys Cys Ile Arg Val Ser Val Gln Leu Glu Ala Ser Pro  
 820 825 830  
 Ala Phe Leu Ala Val Pro Val Glu Lys Glu Gln Ala Pro His Cys Ile  
 835 840 845  
 Cys Ala Asn Gly Arg Gln Thr Val Ser Trp Ala Val Thr Pro Lys Ser  
 850 855 860  
 Leu Gly Asn Val Asn Phe Thr Val Ser Ala Glu Ala Leu Glu Ser Gln  
 865 870 875 880  
 Glu Leu Cys Gly Thr Glu Val Pro Ser Val Pro Glu His Gly Arg Lys  
 885 890 895  
 Asp Thr Val Ile Lys Pro Leu Leu Val Glu Pro Glu Gly Leu Glu Lys  
 900 905 910  
 Glu Thr Thr Phe Asn Ser Leu Leu Cys Pro Ser Gly Gly Glu Val Ser  
 915 920 925  
 Glu Glu Leu Ser Leu Lys Leu Pro Pro Asn Val Val Glu Glu Ser Ala  
 930 935 940  
 Arg Ala Ser Val Ser Val Leu Gly Asp Ile Leu Gly Ser Ala Met Gln  
 945 950 955 960  
 Asn Thr Gln Asn Leu Leu Gln Met Pro Tyr Gly Cys Gly Glu Gln Asn  
 965 970 975  
 Met Val Leu Phe Ala Pro Asn Ile Tyr Val Leu Asp Tyr Leu Asn Glu  
 980 985 990

Thr Gln Gln Leu Thr Pro Glu Ile Lys Ser Lys Ala Ile Gly Tyr Leu  
 995 1000 1005  
 Asn Thr Gly Tyr Gln Arg Gln Leu Asn Tyr Lys His Tyr Asp Gly Ser  
 1010 1015 1020  
 Tyr Ser Thr Phe Gly Glu Arg Tyr Gly Arg Asn Gln Gly Asn Thr Trp  
 1025 1030 1035 1040  
 Leu Thr Ala Phe Val Leu Lys Thr Phe Ala Gln Ala Arg Ala Tyr Ile  
 1045 1050 1055  
 Phe Ile Asp Glu Ala His Ile Thr Gln Ala Leu Ile Trp Leu Ser Gln  
 1060 1065 1070  
 Arg Gln Lys Asp Asn Gly Cys Phe Arg Ser Ser Gly Ser Leu Leu Asn  
 1075 1080 1085  
 Asn Ala Ile Lys Gly Gly Val Glu Asp Glu Val Thr Leu Ser Ala Tyr  
 1090 1095 1100  
 Ile Thr Ile Ala Leu Leu Glu Ile Pro Leu Thr Val Thr His Pro Val  
 1105 1110 1115 1120  
 Val Arg Asn Ala Leu Phe Cys Leu Glu Ser Ala Trp Lys Thr Ala Gln  
 1125 1130 1135  
 Glu Gly Asp His Gly Ser His Val Tyr Thr Lys Ala Leu Leu Ala Tyr  
 1140 1145 1150  
 Ala Phe Ala Leu Ala Gly Asn Gln Asp Lys Arg Lys Glu Val Leu Lys  
 1155 1160 1165  
 Ser Leu Asn Glu Glu Ala Val Lys Lys Asp Asn Ser Val His Trp Glu  
 1170 1175 1180  
 Arg Pro Gln Lys Pro Lys Ala Pro Val Gly His Phe Tyr Glu Pro Gln  
 1185 1190 1195 1200  
 Ala Pro Ser Ala Glu Val Glu Met Thr Ser Tyr Val Leu Leu Ala Tyr  
 1205 1210 1215  
 Leu Thr Ala Gln Pro Ala Pro Thr Ser Glu Asp Leu Thr Ser Ala Thr  
 1220 1225 1230  
 Asn Ile Val Lys Trp Ile Thr Lys Gln Gln Asn Ala Gln Gly Gly Phe  
 1235 1240 1245  
 Ser Ser Thr Gln His Thr Val Val Ala Leu His Ala Leu Ser Lys Tyr  
 1250 1255 1260  
 Gly Ala Ala Thr Phe Thr Arg Thr Gly Lys Ala Ala Gln Val Thr Ile  
 1265 1270 1275 1280  
 Gln Ser Ser Gly Thr Phe Ser Ser Lys Phe Gln Val Asp Asn Asn Asn  
 1285 1290 1295

46

Arg Leu Leu Leu Gln Gln Val Ser Leu Pro Glu Leu Pro Gly Glu Tyr  
 1300 1305 1310  
 Ser Met Lys Val Thr Gly Glu Gly Cys Val Tyr Leu Gln Thr Ser Leu  
 1315 1320 1325  
 Lys Tyr Asn Ile Leu Pro Glu Lys Glu Glu Phe Pro Phe Ala Leu Gly  
 1330 1335 1340  
 Val Gln Thr Leu Pro Gln Thr Cys Asp Glu Pro Lys Ala His Thr Ser  
 1345 1350 1355 1360  
 Phe Gln Ile Ser Leu Ser Val Ser Tyr Thr Gly Ser Arg Ser Ala Ser  
 1365 1370 1375  
 Asn Met Ala Ile Val Asp Val Lys Met Val Ser Gly Phe Ile Pro Leu  
 1380 1385 1390  
 Lys Pro Thr Val Lys Met Leu Glu Arg Ser Asn His Val Ser Arg Thr  
 1395 1400 1405  
 Glu Val Ser Ser Asn His Val Leu Ile Tyr Leu Asp Lys Val Ser Asn  
 1410 1415 1420  
 Gln Thr Leu Ser Leu Phe Phe Thr Val Leu Gln Asp Val Pro Val Arg  
 1425 1430 1435 1440  
 Asp Leu Lys Pro Ala Ile Val Lys Val Tyr Asp Tyr Tyr Glu Thr Asp  
 1445 1450 1455  
 Glu Phe Ala Ile Ala Glu Tyr Asn Ala Pro Cys Ser Lys Asp Leu Gly  
 1460 1465 1470  
 Asn Ala

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4599 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: Y

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 29..4480
- (D) OTHER INFORMATION:



## (ix) FEATURE:

- (A) NAME/KEY: insertion seq  
 (B) LOCATION: 2102..2305  
 (D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

|                                                                 |     |
|-----------------------------------------------------------------|-----|
| GTCTCCTCCA GCTCCTTCTT TCTGCAAC ATG GGG AAG AAC AAA CTC CTT CAT  | 52  |
| Met Gly Lys Asn Lys Leu Leu His                                 |     |
| 1 5                                                             |     |
| CCA AGT CTG GTT CTT CTC CTC TTG GTC CTC CTG CCC ACA GAC GCC TCA | 100 |
| Pro Ser Leu Val Leu Leu Leu Val Leu Leu Pro Thr Asp Ala Ser     |     |
| 10 15 20                                                        |     |
| GTC TCT GGA AAA CCG CAG TAT ATG GTT CTG GTC CCC TCC CTG CTC CAC | 148 |
| Val Ser Gly Lys Pro Gln Tyr Met Val Leu Val Pro Ser Leu Leu His |     |
| 25 30 35 40                                                     |     |
| ACT GAG ACC ACT GAG AAG GGC TGT GTC CTT CTG AGC TAC CTG AAT GAG | 196 |
| Thr Glu Thr Thr Glu Lys Gly Cys Val Leu Leu Ser Tyr Leu Asn Glu |     |
| 45 50 55                                                        |     |
| ACA GTG ACT GTA AGT GCT TCC TTG GAG TCT GTC AGG GGA AAC AGG AGC | 244 |
| Thr Val Thr Val Ser Ala Ser Leu Glu Ser Val Arg Gly Asn Arg Ser |     |
| 60 65 70                                                        |     |
| CTC TTC ACT GAC CTG GAG GCG GAG AAT GAC GTA CTC CAC TGT GTC GCC | 292 |
| Leu Phe Thr Asp Leu Glu Ala Glu Asn Asp Val Leu His Cys Val Ala |     |
| 75 80 85                                                        |     |
| TTC GCT GTC CCA AAG TCT TCA TCC AAT GAG GAG GTA ATG TTC CTC ACT | 340 |
| Phe Ala Val Pro Lys Ser Ser Ser Asn Glu Glu Val Met Phe Leu Thr |     |
| 90 95 100                                                       |     |
| GTC CAA GTG AAA GGA CCA ACC CAA GAA TTT AAG AAG CGG ACC ACA GTG | 388 |
| Val Gln Val Lys Gly Pro Thr Gln Glu Phe Lys Lys Arg Thr Thr Val |     |
| 105 110 115 120                                                 |     |
| ATG GTT AAG AAC GAG GAC AGT CTG GTC TTT GTC CAG ACA GAC AAA TCA | 436 |
| Met Val Lys Asn Glu Asp Ser Leu Val Phe Val Gln Thr Asp Lys Ser |     |
| 125 130 135                                                     |     |
| ATC TAC AAA CCA GGG CAG ACA GTG AAA TTT CGT GTT GTC TCC ATG GAT | 484 |
| Ile Tyr Lys Pro Gly Gln Thr Val Lys Phe Arg Val Val Ser Met Asp |     |
| 140 145 150                                                     |     |
| GAA AAC TTT CAC CCC CTG AAT GAG TTG ATT CCA CTA GTA TAC ATT CAG | 532 |
| Glu Asn Phe His Pro Leu Asn Glu Leu Ile Pro Leu Val Tyr Ile Gln |     |
| 155 160 165                                                     |     |
| GAT CCC AAA GGA AAT CGC ATC GCA CAA TGG CAG AGT TTC CAG TTA GAG | 580 |
| Asp Pro Lys Gly Asn Arg Ile Ala Gln Trp Gln Ser Phe Gln Leu Glu |     |
| 170 175 180                                                     |     |

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|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| GGT GGC CTC AAG CAA TTT TCT TTT CCC CTC TCA TCA GAG CCC TTC CAG<br>Gly Gly Leu Lys Gln Phe Ser Phe Pro Leu Ser Ser Glu Pro Phe Gln<br>185 190 195 200 | 628  |
| GGC TCC TAC AAG GTG GTG GTA CAG AAG AAA TCA GGT GGA AGG ACA GAG<br>Gly Ser Tyr Lys Val Val Val Gln Lys Lys Ser Gly Gly Arg Thr Glu<br>205 210 215     | 676  |
| CAC CCT TTC ACC GTG GAG GAA TTT GTT CTT CCC AAG TTT GAA GTA CAA<br>His Pro Phe Thr Val Glu Glu Phe Val Leu Pro Lys Phe Glu Val Gln<br>220 225 230     | 724  |
| GTA ACA GTG CCA AAG ATA ATC ACC ATC TTG GAA GAA GAG ATG AAT GTA<br>Val Thr Val Pro Lys Ile Ile Thr Ile Leu Glu Glu Glu Met Asn Val<br>235 240 245     | 772  |
| TCA GTG TGT GGC CTA TAC ACA TAT GGG AAG CCT GTC CCT GGA CAT GTG<br>Ser Val Cys Gly Leu Tyr Thr Tyr Gly Lys Pro Val Pro Gly His Val<br>250 255 260     | 820  |
| ACT GTG AGC ATT TGC AGA AAG TAT AGT GAC GCT TCC GAC TGC CAC GGT<br>Thr Val Ser Ile Cys Arg Lys Tyr Ser Asp Ala Ser Asp Cys His Gly<br>265 270 275 280 | 868  |
| GAA GAT TCA CAG GCT TTC TGT GAG AAA TTC AGT GGA CAG CTA AAC AGC<br>Glu Asp Ser Gln Ala Phe Cys Glu Lys Phe Ser Gly Gln Leu Asn Ser<br>285 290 295     | 916  |
| CAT GGC TGC TTC TAT CAG CAA GTA AAA ACC AAG GTC TTC CAG CTG AAG<br>His Gly Cys Phe Tyr Gln Gln Val Lys Thr Lys Val Phe Gln Leu Lys<br>300 305 310     | 964  |
| AGG AAG GAG TAT GAA ATG AAA CTT CAC ACT GAG GCC CAG ATC CAA GAA<br>Arg Lys Glu Tyr Glu Met Lys Leu His Thr Glu Ala Gln Ile Gln Glu<br>315 320 325     | 1012 |
| GAA GGA ACA GTG GTG GAA TTG ACT GGA AGG CAG TCC AGT GAA ATC ACA<br>Glu Gly Thr Val Val Glu Leu Thr Gly Arg Gln Ser Ser Glu Ile Thr<br>330 335 340     | 1060 |
| AGA ACC ATA ACC AAA CTC TCA TTT GTG AAA GTG GAC TCA CAC TTT CGA<br>Arg Thr Ile Thr Lys Leu Ser Phe Val Lys Val Asp Ser His Phe Arg<br>345 350 355 360 | 1108 |
| CAG GGA ATT CCC TTC TTT GGG CAG GTG CGC CTA GTA GAT GGG AAA GGC<br>Gln Gly Ile Pro Phe Phe Gly Gln Val Arg Leu Val Asp Gly Lys Gly<br>365 370 375     | 1156 |
| GTC CCT ATA CCA AAT AAA GTC ATA TTC ATC AGA GGA AAT GAA GCA AAC<br>Val Pro Ile Pro Asn Lys Val Ile Phe Ile Arg Gly Asn Glu Ala Asn<br>380 385 390     | 1204 |
| TAT TAC TCC AAT GCT ACC ACG GAT GAG CAT GGC CTT GTA CAG TTC TCT<br>Tyr Tyr Ser Asn Ala Thr Thr Asp Glu His Gly Leu Val Gln Phe Ser<br>395 400 405     | 1252 |

|                   |                   |            |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |      |
|-------------------|-------------------|------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|------|
| ATC<br>Ile<br>410 | AAC<br>Asn        | ACC<br>Thr | ACC<br>Thr        | AAT<br>Asn        | GTT<br>Val        | ATG<br>Met<br>415 | GGT<br>Gly        | ACC<br>Thr        | TCT<br>Ser        | CTT<br>Leu        | ACT<br>Thr<br>420 | GTT<br>Val        | AGG<br>Arg        | GTC<br>Val        | AAT<br>Asn        | 1300 |
| TAC<br>Tyr<br>425 | AAG<br>Lys        | GAT<br>Asp | CGT<br>Arg        | AGT<br>Ser        | CCC<br>Pro<br>430 | TGT<br>Cys        | TAC<br>Tyr        | GGC<br>Gly        | TAC<br>Tyr        | CAG<br>Gln<br>435 | TGG<br>Trp        | GTG<br>Val        | TCA<br>Ser        | GAA<br>Glu        | GAA<br>Glu<br>440 | 1348 |
| CAC<br>His        | GAA<br>Glu        | GAG<br>Glu | GCA<br>Ala        | CAT<br>His<br>445 | CAC<br>His        | ACT<br>Thr        | GCT<br>Ala        | TAT<br>Tyr        | CTT<br>Leu<br>450 | GTG<br>Val        | TTC<br>Phe        | TCC<br>Ser        | CCA<br>Pro        | AGC<br>Ser<br>455 | AAG<br>Lys        | 1396 |
| AGC<br>Ser        | TTT<br>Phe        | GTC<br>Val | CAC<br>His<br>460 | CTT<br>Leu        | GAG<br>Glu        | CCC<br>Pro        | ATG<br>Met        | TCT<br>Ser        | CAT<br>His        | GAA<br>Glu        | CTA<br>Leu        | CCC<br>Pro        | TGT<br>Cys<br>470 | GGC<br>Gly        | CAT<br>His        | 1444 |
| ACT<br>Thr        | CAG<br>Gln<br>475 | ACA<br>Thr | GTC<br>Val        | CAG<br>Gln        | GCA<br>Ala        | CAT<br>His        | TAT<br>Tyr<br>480 | ATT<br>Ile        | CTG<br>Leu        | AAT<br>Asn        | GGA<br>Gly        | GGC<br>Gly<br>485 | ACC<br>Thr        | CTG<br>Leu        | CTG<br>Leu        | 1492 |
| GGG<br>Gly<br>490 | CTG<br>Leu        | AAG<br>Lys | AAG<br>Lys        | CTC<br>Leu        | TCC<br>Ser        | TTC<br>Phe<br>495 | TAT<br>Tyr        | TAT<br>Tyr        | CTG<br>Leu        | ATA<br>Ile        | ATG<br>Met<br>500 | GCA<br>Ala        | AAG<br>Lys        | GGA<br>Gly        | GGC<br>Gly        | 1540 |
| ATT<br>Ile<br>505 | GTC<br>Val        | CGA<br>Arg | ACT<br>Thr        | GGG<br>Gly        | ACT<br>Thr<br>510 | CAT<br>His        | GGA<br>Gly        | CTG<br>Leu        | CTT<br>Leu        | GTG<br>Val<br>515 | AAG<br>Lys        | CAG<br>Gln        | GAA<br>Glu        | GAC<br>Asp        | ATG<br>Met<br>520 | 1588 |
| AAG<br>Lys        | GGC<br>Gly        | CAT<br>His | TTT<br>Phe        | TCC<br>Ser<br>525 | ATC<br>Ile        | TCA<br>Ser        | ATC<br>Ile        | CCT<br>Pro        | GTG<br>Val<br>530 | AAG<br>Lys        | TCA<br>Ser        | GAC<br>Asp        | ATT<br>Ile        | GCT<br>Ala<br>535 | CCT<br>Pro        | 1636 |
| GTC<br>Val        | GCT<br>Ala        | CGG<br>Arg | TTG<br>Leu<br>540 | CTC<br>Leu        | ATC<br>Ile        | TAT<br>Tyr        | GCT<br>Ala        | GTT<br>Val<br>545 | TTA<br>Leu        | CCT<br>Pro        | ACC<br>Thr        | GGG<br>Gly        | GAC<br>Asp<br>550 | GTG<br>Val        | ATT<br>Ile        | 1684 |
| GGG<br>Gly<br>555 | GAT<br>Asp        | TCT<br>Ser | GCA<br>Ala        | AAA<br>Lys        | TAT<br>Tyr        | GAT<br>Asp        | GTT<br>Val<br>560 | GAA<br>Glu        | AAT<br>Asn        | TGT<br>Cys        | CTG<br>Leu        | GCC<br>Ala<br>565 | AAC<br>Asn        | AAG<br>Lys        | GTG<br>Val        | 1732 |
| GAT<br>Asp<br>570 | TTG<br>Leu        | AGC<br>Ser | TTC<br>Phe        | AGC<br>Ser        | CCA<br>Pro        | TCA<br>Ser        | CAA<br>Gln        | AGT<br>Ser        | CTC<br>Leu        | CCA<br>Pro        | GCC<br>Ala        | TCA<br>Ser        | CAC<br>His        | GCC<br>Ala        | CAC<br>His        | 1780 |
| CTG<br>Leu<br>585 | CGA<br>Arg        | GTC<br>Val | ACA<br>Thr        | GCG<br>Ala        | GCT<br>Ala        | CCT<br>Pro        | CAG<br>Gln        | TCC<br>Ser        | GTC<br>Val        | TGC<br>Cys        | GCC<br>Ala        | CTC<br>Leu        | CGT<br>Arg        | GCT<br>Ala        | GTG<br>Val<br>600 | 1828 |
| GAC<br>Asp        | CAA<br>Gln        | AGC<br>Ser | GTG<br>Val<br>605 | CTG<br>Leu        | CTC<br>Leu        | ATG<br>Met        | AAG<br>Lys        | CCT<br>Pro        | GAT<br>Asp<br>610 | GCT<br>Ala        | GAG<br>Glu        | CTC<br>Leu        | TCG<br>Ser        | GCG<br>Ala<br>615 | TCC<br>Ser        | 1876 |
| TCG<br>Ser        | GTT<br>Val        | TAC<br>Tyr | AAC<br>Asn<br>620 | CTG<br>Leu        | CTA<br>Leu        | CCA<br>Pro        | GAA<br>Glu        | AAG<br>Lys<br>625 | GAC<br>Asp        | CTC<br>Leu        | ACT<br>Thr        | GGC<br>Gly        | TTC<br>Phe<br>630 | CCT<br>Pro        | GGG<br>Gly        | 1924 |

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|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| CCT TTG AAT GAC CAG GAC GAT GAA GAC TGC ATC AAT CGT CAT AAT GTC<br>Pro Leu Asn Asp Gln Asp Asp Glu Asp Cys Ile Asn Arg His Asn Val<br>635 640 645     | 1972 |
| TAT ATT AAT GGA ATC ACA TAT ACT CCA GTA TCA AGT ACA AAT GAA AAG<br>Tyr Ile Asn Gly Ile Thr Tyr Thr Pro Val Ser Ser Thr Asn Glu Lys<br>650 655 660     | 2020 |
| GAT ATG TAC AGC TTC CTA GAG GAC ATG GGC TTA AAG GCA TTC ACC AAC<br>Asp Met Tyr Ser Phe Leu Glu Asp Met Gly Leu Lys Ala Phe Thr Asn<br>665 670 675 680 | 2068 |
| TCA AAG ATT CGT AAA CCC AAA ATG TGT CCA CAG CTG CAG TCA GTG TCA<br>Ser Lys Ile Arg Lys Pro Lys Met Cys Pro Gln Leu Gln Ser Val Ser<br>685 690 695     | 2116 |
| GCC GGC GCC GTG GGA CAG GGA TAT TAT GGA GCC GGA CTG GGA GTG GTG<br>Ala Gly Ala Val Gly Gln Gly Tyr Tyr Gly Ala Gly Leu Gly Val Val<br>700 705 710     | 2164 |
| GAG AGG CCT TAT GTG CCT CAG CTG GGT ACC TAT AAT GTG ATC CCT CTG<br>Glu Arg Pro Tyr Val Pro Gln Leu Gly Thr Tyr Asn Val Ile Pro Leu<br>715 720 725     | 2212 |
| AAT AAT GAG CAG AGC TCA GGA CCT GTG CCT GAG ACA GTG AGG AAG TAT<br>Asn Asn Glu Gln Ser Ser Gly Pro Val Pro Glu Thr Val Arg Lys Tyr<br>730 735 740     | 2260 |
| TTC CCT GAG ACA TGG ATC TGG GAT CTG GTG GTG GTG AAT TCC GCG GGT<br>Phe Pro Glu Thr Trp Ile Trp Asp Leu Val Val Val Asn Ser Ala Gly<br>745 750 755 760 | 2308 |
| GTG GCT GAG GTA GGA GTA ACA GTC CCT GAC ACC ATC ACC GAG TGG AAG<br>Val Ala Glu Val Gly Val Thr Val Pro Asp Thr Ile Thr Glu Trp Lys<br>765 770 775     | 2356 |
| GCA GGG GCC TTC TGC CTG TCT GAA GAT GCT GGA CTT GGT ATC TCT TCC<br>Ala Gly Ala Phe Cys Leu Ser Glu Asp Ala Gly Leu Gly Ile Ser Ser<br>780 785 790     | 2404 |
| ACT GCC TCT CTC CGA GCC TTC CAG CCC TTC TTT GTG GAG CTC ACA ATG<br>Thr Ala Ser Leu Arg Ala Phe Gln Pro Phe Phe Val Glu Leu Thr Met<br>795 800 805     | 2452 |
| CCT TAC TCT GTG ATT CGT GGA GAG GCC TTC ACA CTC AAG GCC ACG GTC<br>Pro Tyr Ser Val Ile Arg Gly Glu Ala Phe Thr Leu Lys Ala Thr Val<br>810 815 820     | 2500 |
| CTA AAC TAC CTT CCC AAA TGC ATC CGG GTC AGT GTG CAG CTG GAA GCC<br>Leu Asn Tyr Leu Pro Lys Cys Ile Arg Val Ser Val Gln Leu Glu Ala<br>825 830 835 840 | 2548 |
| TCT CCC GCC TTC CTA GCT GTC CCA GTG GAG AAG GAA CAA GCG CCT CAC<br>Ser Pro Ala Phe Leu Ala Val Pro Val Glu Lys Glu Gln Ala Pro His<br>845 850 855     | 2596 |

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|------------------------------------------------------------------------------------------------------------------------------------|------|
| TGC ATC TGT GCA AAC GGG CGG CAA ACT GTG TCC TGG GCA GTA ACC CCA<br>Cys Ile Cys Ala Asn Gly Arg Gln Thr Val Ser Trp Ala Val Thr Pro | 2644 |
| 860<br>865<br>870                                                                                                                  |      |
| AAG TCA TTA GGA AAT GTG AAT TTC ACT GTG AGC GCA GAG GCA CTA GAG<br>Lys Ser Leu Gly Asn Val Asn Phe Thr Val Ser Ala Glu Ala Leu Glu | 2692 |
| 875<br>880<br>885                                                                                                                  |      |
| TCT CAA GAG CTG TGT GGG ACT GAG GTG CCT TCA GTT CCT GAA CAC GGA<br>Ser Gln Glu Leu Cys Gly Thr Glu Val Pro Ser Val Pro Glu His Gly | 2740 |
| 890<br>895<br>900                                                                                                                  |      |
| AGG AAA GAC ACA GTC ATC AAG CCT CTG TTG GTT GAA CCT GAA GGA CTA<br>Arg Lys Asp Thr Val Ile Lys Pro Leu Leu Val Glu Pro Glu Gly Leu | 2788 |
| 905<br>910<br>915<br>920                                                                                                           |      |
| GAG AAG GAA ACA ACA TTC AAC TCC CTA CTT TGT CCA TCA GGT GGT GAG<br>Glu Lys Glu Thr Thr Phe Asn Ser Leu Leu Cys Pro Ser Gly Gly Glu | 2836 |
| 925<br>930<br>935                                                                                                                  |      |
| GTT TCT GAA GAA TTA TCC CTG AAA CTG CCA CCA AAT GTG GTA GAA GAA<br>Val Ser Glu Glu Leu Ser Leu Lys Leu Pro Pro Asn Val Val Glu Glu | 2884 |
| 940<br>945<br>950                                                                                                                  |      |
| TCT GCC CGA GCT TCT GTC TCA GTT TTG GGA GAC ATA TTA GGC TCT GCC<br>Ser Ala Arg Ala Ser Val Ser Val Leu Gly Asp Ile Leu Gly Ser Ala | 2932 |
| 955<br>960<br>965                                                                                                                  |      |
| ATG CAA AAC ACA CAA AAT CTT CTC CAG ATG CCC TAT GGC TGT GGA GAG<br>Met Gln Asn Thr Gln Asn Leu Leu Gln Met Pro Tyr Gly Cys Gly Glu | 2980 |
| 970<br>975<br>980                                                                                                                  |      |
| CAG AAT ATG GTC CTC TTT GCT CCT AAC ATC TAT GTA CTG GAT TAT CTA<br>Gln Asn Met Val Leu Phe Ala Pro Asn Ile Tyr Val Leu Asp Tyr Leu | 3028 |
| 985<br>990<br>995<br>1000                                                                                                          |      |
| AAT GAA ACA CAG CAG CTT ACT CCA GAG ATC AAG TCC AAG GCC ATT GGC<br>Asn Glu Thr Gln Gln Leu Thr Pro Glu Ile Lys Ser Lys Ala Ile Gly | 3076 |
| 1005<br>1010<br>1015                                                                                                               |      |
| TAT CTC AAC ACT GGT TAC CAG AGA CAG TTG AAC TAC AAA CAC TAT GAT<br>Tyr Leu Asn Thr Gly Tyr Gln Arg Gln Leu Asn Tyr Lys His Tyr Asp | 3124 |
| 1020<br>1025<br>1030                                                                                                               |      |
| GGC TCC TAC AGC ACC TTT GGG GAG CGA TAT GGC AGG AAC CAG GGC AAC<br>Gly Ser Tyr Ser Thr Phe Gly Glu Arg Tyr Gly Arg Asn Gln Gly Asn | 3172 |
| 1035<br>1040<br>1045                                                                                                               |      |
| ACC TGG CTC ACA GCC TTT GTT CTG AAG ACT TTT GCC CAA GCT CGA GCC<br>Thr Trp Leu Thr Ala Phe Val Leu Lys Thr Phe Ala Gln Ala Arg Ala | 3220 |
| 1050<br>1055<br>1060                                                                                                               |      |
| TAC ATC TTC ATC GAT GAA GCA CAC ATT ACC CAA GCC CTC ATA TGG CTC<br>Tyr Ile Phe Ile Asp Glu Ala His Ile Thr Gln Ala Leu Ile Trp Leu | 3268 |
| 1065<br>1070<br>1075<br>1080                                                                                                       |      |

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| TCC CAG AGG CAG AAG GAC AAT GGC TGT TTC AGG AGC TCT GGG TCA CTG<br>Ser Gln Arg Gln Lys Asp Asn Gly Cys Phe Arg Ser Ser Gly Ser Leu<br>1085 1090 1095      | 3316 |
| CTC AAC AAT GCC ATA AAG GGA GGA GTA GAA GAT GAA GTG ACC CTC TCC<br>Leu Asn Asn Ala Ile Lys Gly Gly Val Glu Asp Glu Val Thr Leu Ser<br>1100 1105 1110      | 3364 |
| GCC TAT ATC ACC ATC GCC CTT CTG GAG ATT CCT CTC ACA GTC ACT CAC<br>Ala Tyr Ile Thr Ile Ala Leu Leu Glu Ile Pro Leu Thr Val Thr His<br>1115 1120 1125      | 3412 |
| CCT GTT GTC CGC AAT GCC CTG TTT TGC CTG GAG TCA GCC TGG AAG ACA<br>Pro Val Val Arg Asn Ala Leu Phe Cys Leu Glu Ser Ala Trp Lys Thr<br>1130 1135 1140      | 3460 |
| GCA CAA GAA GGG GAC CAT GGC AGC CAT GTA TAT ACC AAA GCA CTG CTG<br>Ala Gln Glu Gly Asp His Gly Ser His Val Tyr Thr Lys Ala Leu Leu<br>1145 1150 1155 1160 | 3508 |
| GCC TAT GCT TTT GCC CTG GCA GGT AAC CAG GAC AAG AGG AAG GAA GTA<br>Ala Tyr Ala Phe Ala Leu Ala Gly Asn Gln Asp Lys Arg Lys Glu Val<br>1165 1170 1175      | 3556 |
| CTC AAG TCA CTT AAT GAG GAA GCT GTG AAG AAA GAC AAC TCT GTC CAT<br>Leu Lys Ser Leu Asn Glu Glu Ala Val Lys Lys Asp Asn Ser Val His<br>1180 1185 1190      | 3604 |
| TGG GAG CGC CCT CAG AAA CCC AAG GCA CCA GTG GGG CAT TTT TAC GAA<br>Trp Glu Arg Pro Gln Lys Pro Lys Ala Pro Val Gly His Phe Tyr Glu<br>1195 1200 1205      | 3652 |
| CCC CAG GCT CCC TCT GCT GAG GTG GAG ATG ACA TCC TAT GTG CTC CTC<br>Pro Gln Ala Pro Ser Ala Glu Val Glu Met Thr Ser Tyr Val Leu Leu<br>1210 1215 1220      | 3700 |
| GCT TAT CTC ACG GCC CAG CCA GCC CCA ACC TCG GAG GAC CTG ACC TCT<br>Ala Tyr Leu Thr Ala Gln Pro Ala Pro Thr Ser Glu Asp Leu Thr Ser<br>1225 1230 1235 1240 | 3748 |
| GCA ACC AAC ATC GTG AAG TGG ATC ACG AAG CAG CAG AAT GCC CAG GGC<br>Ala Thr Asn Ile Val Lys Trp Ile Thr Lys Gln Gln Asn Ala Gln Gly<br>1245 1250 1255      | 3796 |
| GGT TTC TCC TCC ACC CAG CAC ACA GTG GTG GCT CTC CAT GCT CTG TCC<br>Gly Phe Ser Ser Thr Gln His Thr Val Val Ala Leu His Ala Leu Ser<br>1260 1265 1270      | 3844 |
| AAA TAT GGA GCA GCC ACA TTT ACC AGG ACT GGG AAG GCT GCA CAG GTG<br>Lys Tyr Gly Ala Ala Thr Phe Thr Arg Thr Gly Lys Ala Ala Gln Val<br>1275 1280 1285      | 3892 |
| ACT ATC CAG TCT TCA GGG ACA TTT TCC AGC AAA TTC CAA GTG GAC AAC<br>Thr Ile Gln Ser Ser Gly Thr Phe Ser Ser Lys Phe Gln Val Asp Asn<br>1290 1295 1300      | 3940 |

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| AAC AAC CGC CTG TTA CTG CAG CAG GTC TCA TTG CCA GAG CTG CCT GGG<br>Asn Asn Arg Leu Leu Leu Gln Gln Val Ser Leu Pro Glu Leu Pro Gly<br>1305 1310 1315 1320 | 3988 |
| GAA TAC AGC ATG AAA GTG ACA GGA GAA GGA TGT GTC TAC CTC CAG ACA<br>Glu Tyr Ser Met Lys Val Thr Gly Glu Gly Cys Val Tyr Leu Gln Thr<br>1325 1330 1335      | 4036 |
| TCC TTG AAA TAC AAT ATT CTC CCA GAA AAG GAA GAG TTC CCC TTT GCT<br>Ser Leu Lys Tyr Asn Ile Leu Pro Glu Lys Glu Glu Phe Pro Phe Ala<br>1340 1345 1350      | 4084 |
| TTA GGA GTG CAG ACT CTG CCT CAA ACT TGT GAT GAA CCC AAA GCC CAC<br>Leu Gly Val Gln Thr Leu Pro Gln Thr Cys Asp Glu Pro Lys Ala His<br>1355 1360 1365      | 4132 |
| ACC AGC TTC CAA ATC TCC CTA AGT GTC AGT TAC ACA GGG AGC CGC TCT<br>Thr Ser Phe Gln Ile Ser Leu Ser Val Ser Tyr Thr Gly Ser Arg Ser<br>1370 1375 1380      | 4180 |
| GCC TCC AAC ATG GCG ATC GTT GAT GTG AAG ATG GTC TCT GGC TTC ATT<br>Ala Ser Asn Met Ala Ile Val Asp Val Lys Met Val Ser Gly Phe Ile<br>1385 1390 1395 1400 | 4228 |
| CCC CTG AAG CCA ACA GTG AAA ATG CTT GAA AGA TCT AAC CAT GTG AGC<br>Pro Leu Lys Pro Thr Val Lys Met Leu Glu Arg Ser Asn His Val Ser<br>1405 1410 1415      | 4276 |
| CGG ACA GAA GTC AGC AGC AAC CAT GTC TTG ATT TAC CTT GAT AAG GTG<br>Arg Thr Glu Val Ser Ser Asn His Val Leu Ile Tyr Leu Asp Lys Val<br>1420 1425 1430      | 4324 |
| TCA AAT CAG ACA CTG AGC TTG TTC TTC ACG GTT CTG CAA GAT GTC CCA<br>Ser Asn Gln Thr Leu Ser Leu Phe Phe Thr Val Leu Gln Asp Val Pro<br>1435 1440 1445      | 4372 |
| GTA AGA GAT CTG AAA CCA GCC ATA GTG AAA GTC TAT GAT TAC TAC GAG<br>Val Arg Asp Leu Lys Pro Ala Ile Val Lys Val Tyr Asp Tyr Tyr Glu<br>1450 1455 1460      | 4420 |
| ACG GAT GAG TTT GCA ATT GCT GAG TAC AAT GCT CCT TGC AGC AAA GAT<br>Thr Asp Glu Phe Ala Ile Ala Glu Tyr Asn Ala Pro Cys Ser Lys Asp<br>1465 1470 1475 1480 | 4468 |
| CTT GGA AAT GCT TGAAGACCAC AAGGCTGAAA AGTGCTTTGC TGGAGTCCTG<br>Leu Gly Asn Ala                                                                            | 4520 |
| TTCTCTGAGC TCCACAGAAG ACACGTGTTT TTGTATCTTT AAAGACTTGA TGAATAAACA                                                                                         | 4580 |
| CTTTTTCTGG TCAAAAAA                                                                                                                                       | 4599 |

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1484 amino acids

(B) TYPE: amino acid

54

(D) TOPOLOGY: linear

(E) FEATURES: bait region: 690-740

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Gly Lys Asn Lys Leu Leu His Pro Ser Leu Val Leu Leu Leu Leu
 1 5 10 15
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 20 25 30
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 35 40 45
Val Leu Leu Ser Tyr Leu Asn Glu Thr Val Thr Val Ser Ala Ser Leu
 50 55 60
Glu Ser Val Arg Gly Asn Arg Ser Leu Phe Thr Asp Leu Glu Ala Glu
 65 70 75 80
Asn Asp Val Leu His Cys Val Ala Phe Ala Val Pro Lys Ser Ser Ser
 85 90 95
Asn Glu Glu Val Met Phe Leu Thr Val Gln Val Lys Gly Pro Thr Gln
 100 105 110
Glu Phe Lys Lys Arg Thr Thr Val Met Val Lys Asn Glu Asp Ser Leu
 115 120 125
Val Phe Val Gln Thr Asp Lys Ser Ile Tyr Lys Pro Gly Gln Thr Val
 130 135 140
Lys Phe Arg Val Val Ser Met Asp Glu Asn Phe His Pro Leu Asn Glu
 145 150 155 160
Leu Ile Pro Leu Val Tyr Ile Gln Asp Pro Lys Gly Asn Arg Ile Ala
 165 170 175
Gln Trp Gln Ser Phe Gln Leu Glu Gly Gly Leu Lys Gln Phe Ser Phe
 180 185 190
Pro Leu Ser Ser Glu Pro Phe Gln Gly Ser Tyr Lys Val Val Val Gln
 195 200 205
Lys Lys Ser Gly Gly Arg Thr Glu His Pro Phe Thr Val Glu Glu Phe
 210 215 220
Val Leu Pro Lys Phe Glu Val Gln Val Thr Val Pro Lys Ile Ile Thr
 225 230 235 240
Ile Leu Glu Glu Glu Met Asn Val Ser Val Cys Gly Leu Tyr Thr Tyr
 245 250 255
Gly Lys Pro Val Pro Gly His Val Thr Val Ser Ile Cys Arg Lys Tyr
 260 265 270

```



55

Ser Asp Ala Ser Asp Cys His Gly Glu Asp Ser Gln Ala Phe Cys Glu  
 275 280 285  
 Lys Phe Ser Gly Gln Leu Asn Ser His Gly Cys Phe Tyr Gln Gln Val  
 290 295 300  
 Lys Thr Lys Val Phe Gln Leu Lys Arg Lys Glu Tyr Glu Met Lys Leu  
 305 310 315 320  
 His Thr Glu Ala Gln Ile Gln Glu Glu Gly Thr Val Val Glu Leu Thr  
 325 330 335  
 Gly Arg Gln Ser Ser Glu Ile Thr Arg Thr Ile Thr Lys Leu Ser Phe  
 340 345 350  
 Val Lys Val Asp Ser His Phe Arg Gln Gly Ile Pro Phe Phe Gly Gln  
 355 360 365  
 Val Arg Leu Val Asp Gly Lys Gly Val Pro Ile Pro Asn Lys Val Ile  
 370 375 380  
 Phe Ile Arg Gly Asn Glu Ala Asn Tyr Tyr Ser Asn Ala Thr Thr Asp  
 385 390 395 400  
 Glu His Gly Leu Val Gln Phe Ser Ile Asn Thr Thr Asn Val Met Gly  
 405 410 415  
 Thr Ser Leu Thr Val Arg Val Asn Tyr Lys Asp Arg Ser Pro Cys Tyr  
 420 425 430  
 Gly Tyr Gln Trp Val Ser Glu Glu His Glu Glu Ala His His Thr Ala  
 435 440 445  
 Tyr Leu Val Phe Ser Pro Ser Lys Ser Phe Val His Leu Glu Pro Met  
 450 455 460  
 Ser His Glu Leu Pro Cys Gly His Thr Gln Thr Val Gln Ala His Tyr  
 465 470 475 480  
 Ile Leu Asn Gly Gly Thr Leu Leu Gly Leu Lys Lys Leu Ser Phe Tyr  
 485 490 495  
 Tyr Leu Ile Met Ala Lys Gly Gly Ile Val Arg Thr Gly Thr His Gly  
 500 505 510  
 Leu Leu Val Lys Gln Glu Asp Met Lys Gly His Phe Ser Ile Ser Ile  
 515 520 525  
 Pro Val Lys Ser Asp Ile Ala Pro Val Ala Arg Leu Leu Ile Tyr Ala  
 530 535 540  
 Val Leu Pro Thr Gly Asp Val Ile Gly Asp Ser Ala Lys Tyr Asp Val  
 545 550 555 560  
 Glu Asn Cys Leu Ala Asn Lys Val Asp Leu Ser Phe Ser Pro Ser Gln  
 565 570 575

56

Ser Leu Pro Ala Ser His Ala His Leu Arg Val Thr Ala Ala Pro Gln  
 580 585 590  
 Ser Val Cys Ala Leu Arg Ala Val Asp Gln Ser Val Leu Leu Met Lys  
 595 600 605  
 Pro Asp Ala Glu Leu Ser Ala Ser Ser Val Tyr Asn Leu Leu Pro Glu  
 610 615 620  
 Lys Asp Leu Thr Gly Phe Pro Gly Pro Leu Asn Asp Gln Asp Asp Glu  
 625 630 635 640  
 Asp Cys Ile Asn Arg His Asn Val Tyr Ile Asn Gly Ile Thr Tyr Thr  
 645 650 655  
 Pro Val Ser Ser Thr Asn Glu Lys Asp Met Tyr Ser Phe Leu Glu Asp  
 660 665 670  
 Met Gly Leu Lys Ala Phe Thr Asn Ser Lys Ile Arg Lys Pro Lys Met  
 675 680 685  
 Cys Pro Gln Leu Gln Ser Val Ser Ala Gly Ala Val Gly Gln Gly Tyr  
 690 695 700  
 Tyr Gly Ala Gly Leu Gly Val Val Glu Arg Pro Tyr Val Pro Gln Leu  
 705 710 715 720  
 Gly Thr Tyr Asn Val Ile Pro Leu Asn Asn Glu Gln Ser Ser Gly Pro  
 725 730 735  
 Val Pro Glu Thr Val Arg Lys Tyr Phe Pro Glu Thr Trp Ile Trp Asp  
 740 745 750  
 Leu Val Val Val Asn Ser Ala Gly Val Ala Glu Val Gly Val Thr Val  
 755 760 765  
 Pro Asp Thr Ile Thr Glu Trp Lys Ala Gly Ala Phe Cys Leu Ser Glu  
 770 775 780  
 Asp Ala Gly Leu Gly Ile Ser Ser Thr Ala Ser Leu Arg Ala Phe Gln  
 785 790 795 800  
 Pro Phe Phe Val Glu Leu Thr Met Pro Tyr Ser Val Ile Arg Gly Glu  
 805 810 815  
 Ala Phe Thr Leu Lys Ala Thr Val Leu Asn Tyr Leu Pro Lys Cys Ile  
 820 825 830  
 Arg Val Ser Val Gln Leu Glu Ala Ser Pro Ala Phe Leu Ala Val Pro  
 835 840 845  
 Val Glu Lys Glu Gln Ala Pro His Cys Ile Cys Ala Asn Gly Arg Gln  
 850 855 860  
 Thr Val Ser Trp Ala Val Thr Pro Lys Ser Leu Gly Asn Val Asn Phe  
 865 870 875 880

57

Thr Val Ser Ala Glu Ala Leu Glu Ser Gln Glu Leu Cys Gly Thr Glu  
 885 890 895  
 Val Pro Ser Val Pro Glu His Gly Arg Lys Asp Thr Val Ile Lys Pro  
 900 905 910  
 Leu Leu Val Glu Pro Glu Gly Leu Glu Lys Glu Thr Thr Phe Asn Ser  
 915 920 925  
 Leu Leu Cys Pro Ser Gly Gly Glu Val Ser Glu Glu Leu Ser Leu Lys  
 930 935 940  
 Leu Pro Pro Asn Val Val Glu Glu Ser Ala Arg Ala Ser Val Ser Val  
 945 950 955 960  
 Leu Gly Asp Ile Leu Gly Ser Ala Met Gln Asn Thr Gln Asn Leu Leu  
 965 970 975  
 Gln Met Pro Tyr Gly Cys Gly Glu Gln Asn Met Val Leu Phe Ala Pro  
 980 985 990  
 Asn Ile Tyr Val Leu Asp Tyr Leu Asn Glu Thr Gln Gln Leu Thr Pro  
 995 1000 1005  
 Glu Ile Lys Ser Lys Ala Ile Gly Tyr Leu Asn Thr Gly Tyr Gln Arg  
 1010 1015 1020  
 Gln Leu Asn Tyr Lys His Tyr Asp Gly Ser Tyr Ser Thr Phe Gly Glu  
 1025 1030 1035 1040  
 Arg Tyr Gly Arg Asn Gln Gly Asn Thr Trp Leu Thr Ala Phe Val Leu  
 1045 1050 1055  
 Lys Thr Phe Ala Gln Ala Arg Ala Tyr Ile Phe Ile Asp Glu Ala His  
 1060 1065 1070  
 Ile Thr Gln Ala Leu Ile Trp Leu Ser Gln Arg Gln Lys Asp Asn Gly  
 1075 1080 1085  
 Cys Phe Arg Ser Ser Gly Ser Leu Leu Asn Asn Ala Ile Lys Gly Gly  
 1090 1095 1100  
 Val Glu Asp Glu Val Thr Leu Ser Ala Tyr Ile Thr Ile Ala Leu Leu  
 1105 1110 1115 1120  
 Glu Ile Pro Leu Thr Val Thr His Pro Val Val Arg Asn Ala Leu Phe  
 1125 1130 1135  
 Cys Leu Glu Ser Ala Trp Lys Thr Ala Gln Glu Gly Asp His Gly Ser  
 1140 1145 1150  
 His Val Tyr Thr Lys Ala Leu Leu Ala Tyr Ala Phe Ala Leu Ala Gly  
 1155 1160 1165  
 Asn Gln Asp Lys Arg Lys Glu Val Leu Lys Ser Leu Asn Glu Glu Ala  
 1170 1175 1180

58

Val Lys Lys Asp Asn Ser Val His Trp Glu Arg Pro Gln Lys Pro Lys  
1185 1190 1195 1200

Ala Pro Val Gly His Phe Tyr Glu Pro Gln Ala Pro Ser Ala Glu Val  
1205 1210 1215

Glu Met Thr Ser Tyr Val Leu Leu Ala Tyr Leu Thr Ala Gln Pro Ala  
1220 1225 1230

Pro Thr Ser Glu Asp Leu Thr Ser Ala Thr Asn Ile Val Lys Trp Ile  
1235 1240 1245

Thr Lys Gln Gln Asn Ala Gln Gly Gly Phe Ser Ser Thr Gln His Thr  
1250 1255 1260

Val Val Ala Leu His Ala Leu Ser Lys Tyr Gly Ala Ala Thr Phe Thr  
1265 1270 1275 1280

Arg Thr Gly Lys Ala Ala Gln Val Thr Ile Gln Ser Ser Gly Thr Phe  
1285 1290 1295

Ser Ser Lys Phe Gln Val Asp Asn Asn Asn Arg Leu Leu Leu Gln Gln  
1300 1305 1310

Val Ser Leu Pro Glu Leu Pro Gly Glu Tyr Ser Met Lys Val Thr Gly  
1315 1320 1325

Glu Gly Cys Val Tyr Leu Gln Thr Ser Leu Lys Tyr Asn Ile Leu Pro  
1330 1335 1340

Glu Lys Glu Glu Phe Pro Phe Ala Leu Gly Val Gln Thr Leu Pro Gln  
1345 1350 1355 1360

Thr Cys Asp Glu Pro Lys Ala His Thr Ser Phe Gln Ile Ser Leu Ser  
1365 1370 1375

Val Ser Tyr Thr Gly Ser Arg Ser Ala Ser Asn Met Ala Ile Val Asp  
1380 1385 1390

Val Lys Met Val Ser Gly Phe Ile Pro Leu Lys Pro Thr Val Lys Met  
1395 1400 1405

Leu Glu Arg Ser Asn His Val Ser Arg Thr Glu Val Ser Ser Asn His  
1410 1415 1420

Val Leu Ile Tyr Leu Asp Lys Val Ser Asn Gln Thr Leu Ser Leu Phe  
1425 1430 1435 1440

Phe Thr Val Leu Gln Asp Val Pro Val Arg Asp Leu Lys Pro Ala Ile  
1445 1450 1455

Val Lys Val Tyr Asp Tyr Tyr Glu Thr Asp Glu Phe Ala Ile Ala Glu  
1460 1465 1470

Tyr Asn Ala Pro Cys Ser Lys Asp Leu Gly Asn Ala  
1475 1480

PATENT CLAIMS

1. A process for the production of recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof, wherein a functionally operative expression vector comprising a gene encoding for the expression of  $\alpha$ -  
5 macroglobulin, variants, fragments or derivatives thereof, or alleles of such a gene, is introduced into a suitable host capable of expressing said gene, said host is cultured in a suitable nutrient medium containing sources of assimilable carbon and nitrogen and other essential nutrients, and the expressed  $\alpha$ -macroglobulin or fragments or derivatives thereof is recovered.  
10
2. The process of claim 1, wherein said gene encodes for the expression of human  $\alpha_2$ -macroglobulin, variants, fragments or derivatives thereof.
- 15 3. The process of claim 2, wherein said gene encodes for the expression of human  $\alpha_2$ -macroglobulin having the amino acid sequence of SEQ ID NO:2, or a fragment or derivative thereof.
4. The process of claim 2 or 3, wherein said gene comprises the DNA  
20 sequence of SEQ ID NO:1, or a fragment thereof.
5. The process of claim 1 or 2, wherein said gene encodes for a variant  $\alpha$ -macroglobulin, in which the amino acid sequence of the bait region has been altered.  
25
6. The process of claim 5, wherein the bait region has been altered by incorporation of further proteinase target sites.
7. The process of claim 5, wherein the bait region has been altered  
30 by removal of proteinase target sites.
8. The process of claim 5, wherein the bait region has been altered by replacing one or more specific proteinase target sites with one or more other specific proteinase target sites.  
35
9. The process of claim 8, wherein said proteinase target sites are specific for bovine trypsin, Streptomyces griseus trypsin, papain, porcine elastase, bovine chymosin, bovine chymotrypsin, Staphylococcus aureus strain

V8 proteinase, human plasmin, bovine thrombin, thermolysin, subtilisin Novo and/or Streptomyces griseus proteinase B.

10. The process of claim 5, wherein wherein the bait region has been  
5 altered by replacing said bait region or part thereof with a bait region or  
a part thereof from another  $\alpha$ -macroglobulin.

11. The process of claim 10, wherein said bait regions originate from  
human  $\alpha_2$ M, Pregnancy Zone Protein (PZP), rat  $\alpha_1$ M, rat  $\alpha_2$ M, rat  $\alpha_1$ I<sub>3</sub> variant  
10 1, or rat  $\alpha_1$ I<sub>3</sub> variant 2 ( $\alpha_1$ I<sub>3</sub> =  $\alpha_1$ -inhibitor 3), especially PZP.

12. The process of any of claims 5 to 11, wherein said gene encodes  
for the expression of human  $\alpha_2$ -macroglobulin variant having the amino acid  
sequence of SEQ ID NO:4, or a fragment or derivative thereof.

15 13. The process of any of claims 5 to 12, wherein said gene comprises  
the DNA sequence of SEQ ID NO:3, or a fragment thereof.

14. The process of any of the claims 1 to 13, wherein said gene is  
20 a synthetic gene.

15. The process of any of the claims 1 to 14, wherein said  $\alpha$ -  
macroglobulin, variant, fragment or derivative thereof is co-expressed with  
a desired gene product.

25 16. The process of any of the claims 1 to 15, wherein said gene is,  
or is derived from, a human gene.

17. The process of any of the claims 1 to 16, wherein said host is  
30 a bacterial strain, a fungal strain, a mammalian cell line, or a mammal.

18. The process of claim 17, wherein said host is a fungus.

19. The process of claim 18, wherein said fungus belongs to the genus  
35 Aspergillus.

20 The process of claim 18, wherein said host is a yeast.

21. The process of claim 20, wherein said yeast belongs to the genus Saccharomyces.
22. The process of claim 17, wherein said host is a mammalian cell  
5 line.
23. The process of claim 22, wherein said mammalian cell line is a Syrian Baby Hamster Kidney (BKH) cell line.
- 10 24. The process of claim 23, wherein said cell line is available from ATCC under No. CRL 1632.
25. A DNA sequence comprising a gene encoding for the expression of an  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof.  
15
- 26 The DNA sequence of claim 25, wherein said gene encodes for human  $\alpha_2$ -macroglobulin.
27. The DNA sequence of claim 25, wherein said gene encodes for the amino  
20 acid sequence of SEQ ID NO:2 or a fragment or derivative thereof.
28. The DNA sequence of claim 26 or 27, wherein said gene has the nucleotide sequence of SEQ ID NO:1 or a fragment thereof.
- 25 29. The DNA sequence of claim 25 or 26, wherein said gene encodes for a variant  $\alpha$ -macroglobulin, in which the amino acid sequence of the bait region has been altered.
30. The DNA sequence of claim 29, wherein said bait region has been  
30 altered by incorporation of further proteinase target sites.
31. The DNA sequence of claim 29, wherein said bait region has been altered by removal of proteinase target sites.
- 35 32. The DNA sequence of claim 29, wherein said bait region has been altered by replacing one or more specific proteinase target sites with one or more other specific proteinase target sites.

33. The DNA sequence of claim 29, wherein, wherein said proteinase target sites are specific for bovine trypsin, Streptomyces griseus trypsin, papain, porcine elastase, bovine chymosin, bovine chymotrypsin, Staphylococcus aureus strain V8 proteinase, human plasmin, bovine thrombin, thermolysin, subtilisin Novo and/or Streptomyces griseus proteinase B.

34. The DNA sequence of claim 29, wherein the bait region has been altered by replacing said bait region or part thereof with a bait region or a part thereof from another  $\alpha$ -macroglobulin.

35. The DNA sequence of claim 34, wherein said bait region originates from human  $\alpha_2$ M, Pregnancy Zone Protein (PZP), rat  $\alpha_1$ M, rat  $\alpha_2$ M, rat  $\alpha_1$ I<sub>3</sub> variant 1, or rat  $\alpha_1$ I<sub>3</sub> variant 2, especially PZP.

36. A functionally operative expression vector comprising a gene in accordance with any of the claims 25 to 35 for the expression of human  $\alpha_2$ -macroglobulin, variants, fragments or derivatives thereof, or alleles of such a gene.

37. The vector of claim 36, further comprising regulatory elements necessary for the stable maintenance of said vector in mammalian cells.

38. The vector of claim 36 or 37, further comprising sequences providing for the processing and secretion of the expressed product.

39. The vector of any of the claims 36 to 38, further comprising one or more other genes encoding for a desired gene product.

40. A functionally operative expression vector comprising a gene encoding for the expression of an  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof, or alleles of such a gene, essentially as described.

41. A transformed host comprising a functionally operative expression vector comprising a gene encoding for the expression of human  $\alpha_2$ -macroglobulin or fragments or derivatives thereof, or alleles of such a gene.

42. The host of claim 41, wherein said vector is the vector of any of the claims 36 to 40.



43. The host of claim 41 or 42, wherein said host is a bacterial strain, a fungal strain, a mammalian cell line, or a mammal.
44. The host of claim 43, wherein said host is a fungus.
45. The host of claim 44, wherein said fungus belongs to the genus Aspergillus.
46. The host of claim 44, wherein said host is a yeast.
47. The host of claim 46, wherein said host belongs to the genus Saccharomyces.
48. The host of claim 43, wherein said host is a mammalian cell line.
49. The host of claim 48, wherein said host is a Syrian Baby Hamster Kidney (BHK) cell line.
50. The host of claim 49, wherein said cell line is available from ATCC under No. CRL 1632.
51. Recombinant human  $\alpha_2$ -macroglobulin of SEQ ID NO:2 or SEQ ID NO:4 in an active form.
52. Recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof produced by a process of any of the claims 1 to 24.
53. Recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof of claim 52 produced by the use of a vector of any of the claims 36 to 40.
54. Recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof essentially as described.
55. Recombinant human  $\alpha_2$ -macroglobulin, variants, fragments or derivatives thereof essentially as described.
56. A growth medium comprising one or more  $\alpha$ -macroglobulins.

57. A growth medium comprising recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof according to any of the claims 51 to 55.

58. Use of recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof according to any of the claims 51 to 55 as a protein carrier in enzyme replacement therapy.

59. Use of recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof according to any of the claims 51 to 55 as a DNA carrier in gene therapy.

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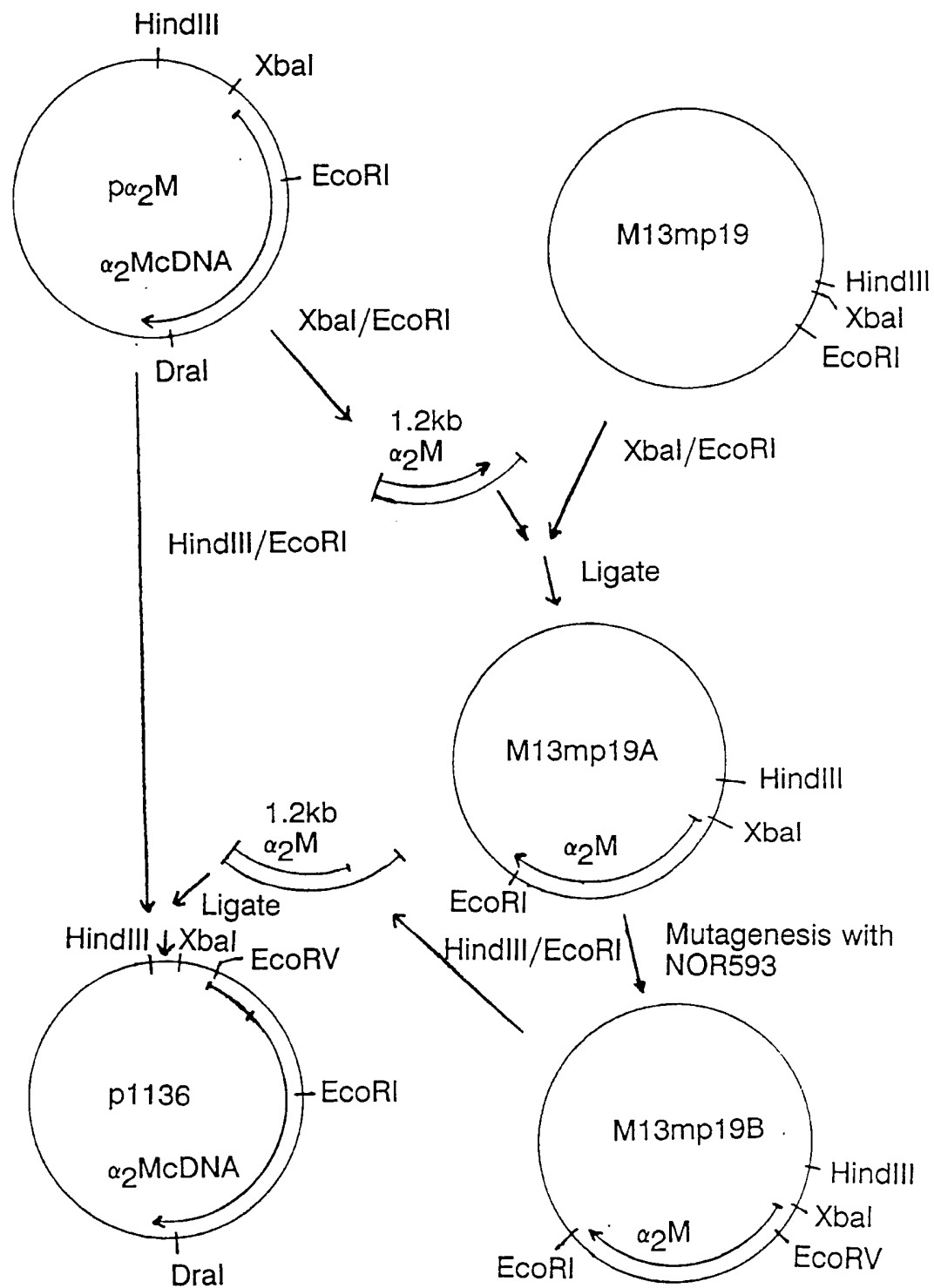


FIG. 1A

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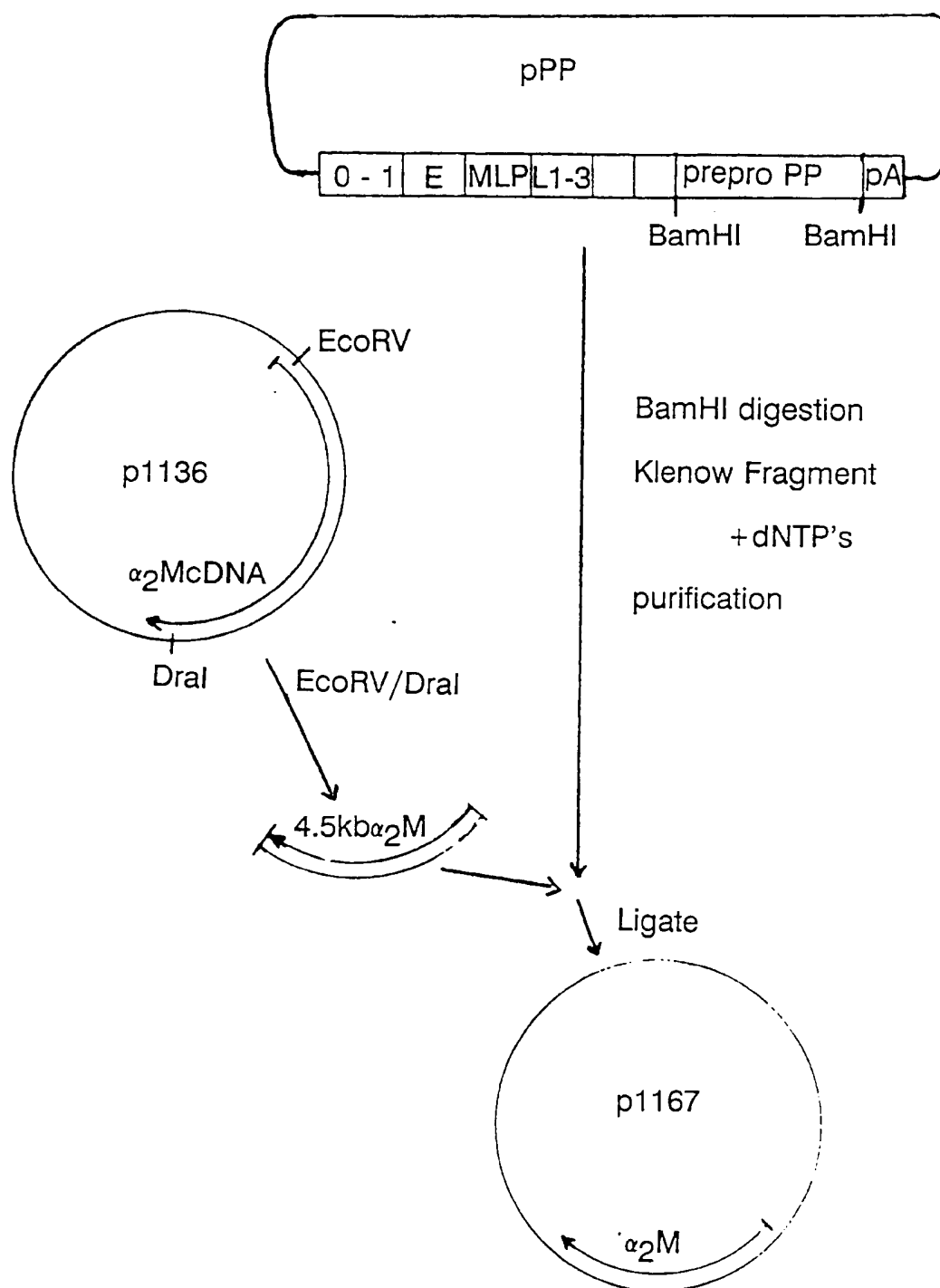


FIG. 1B

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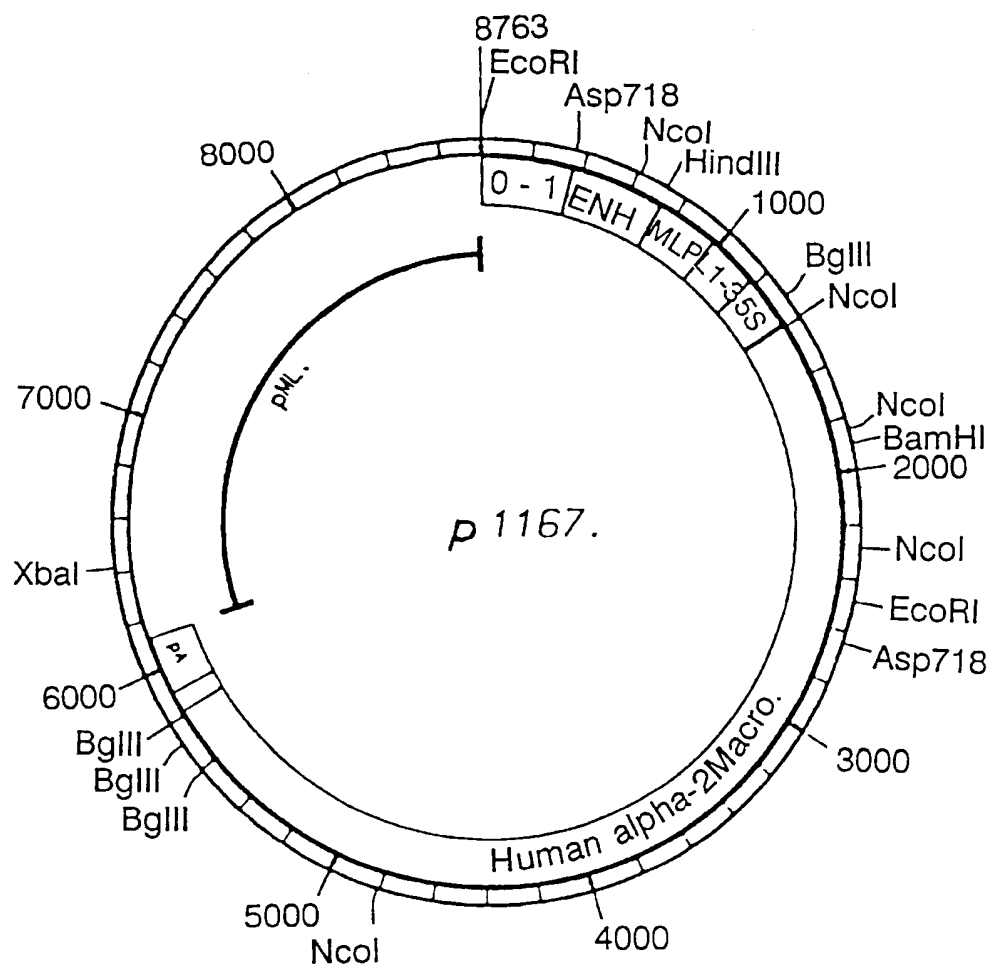


FIG. 2

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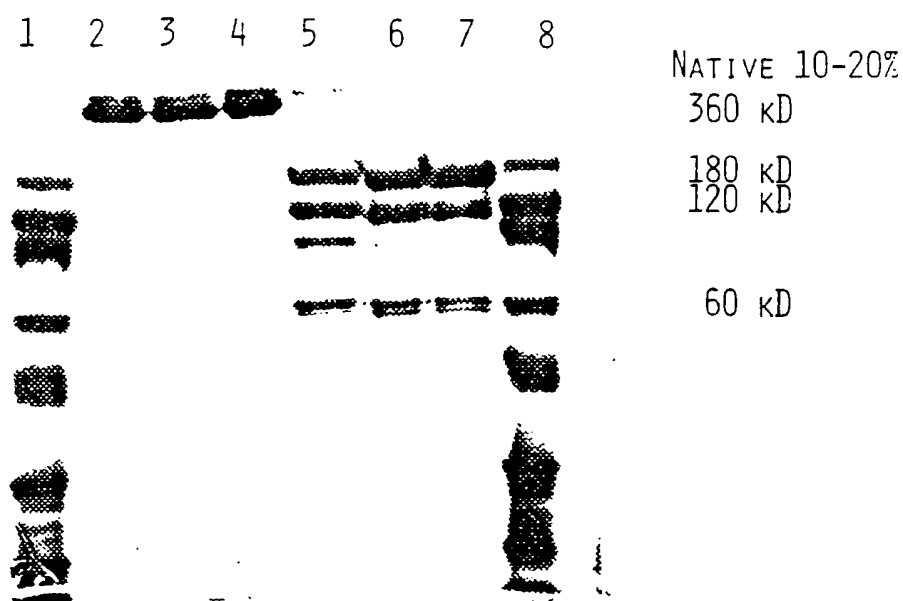


FIG. 3

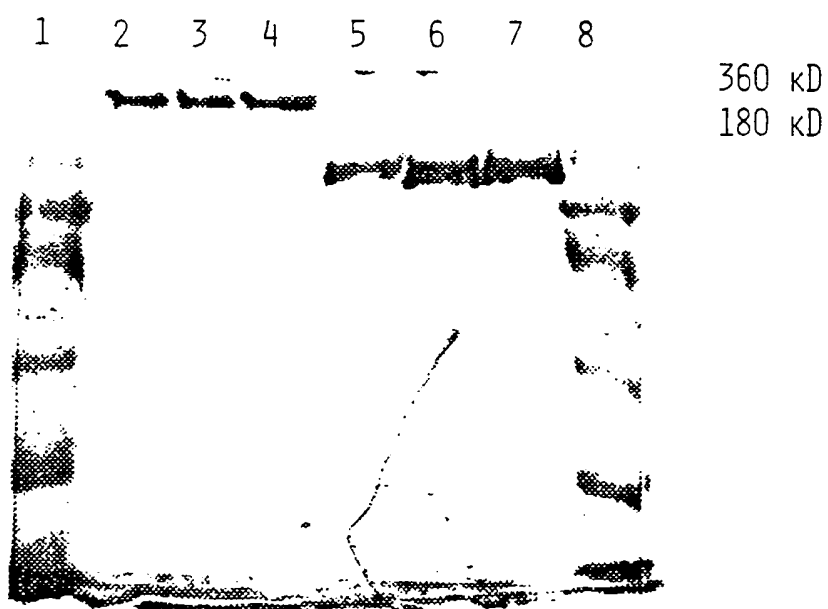


FIG. 4

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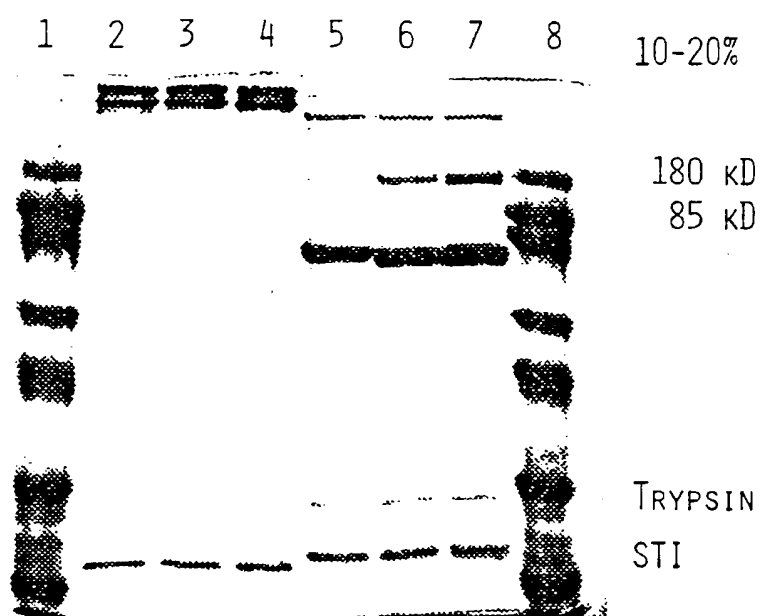


FIG. 5

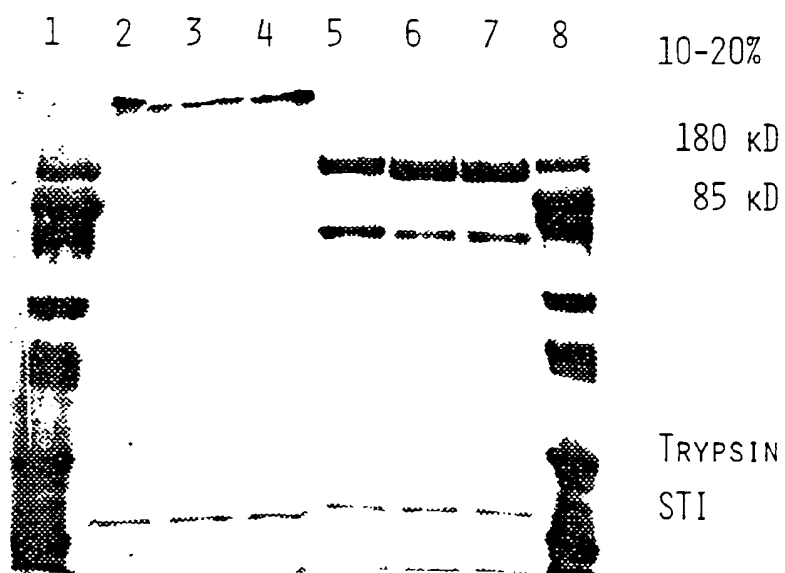


FIG. 6

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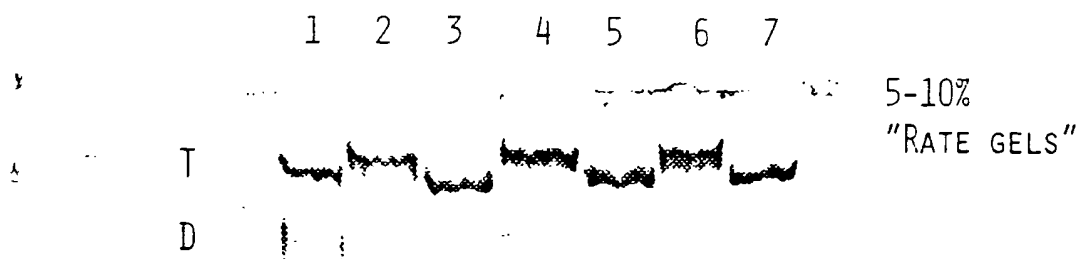


FIG. 7

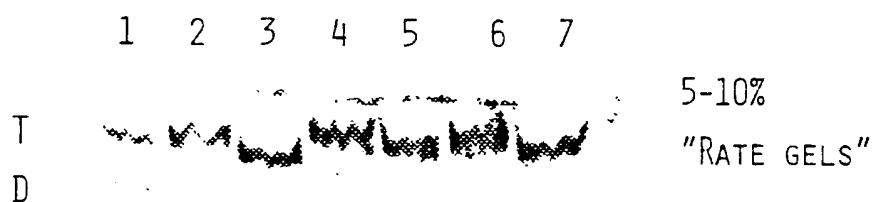


FIG. 8



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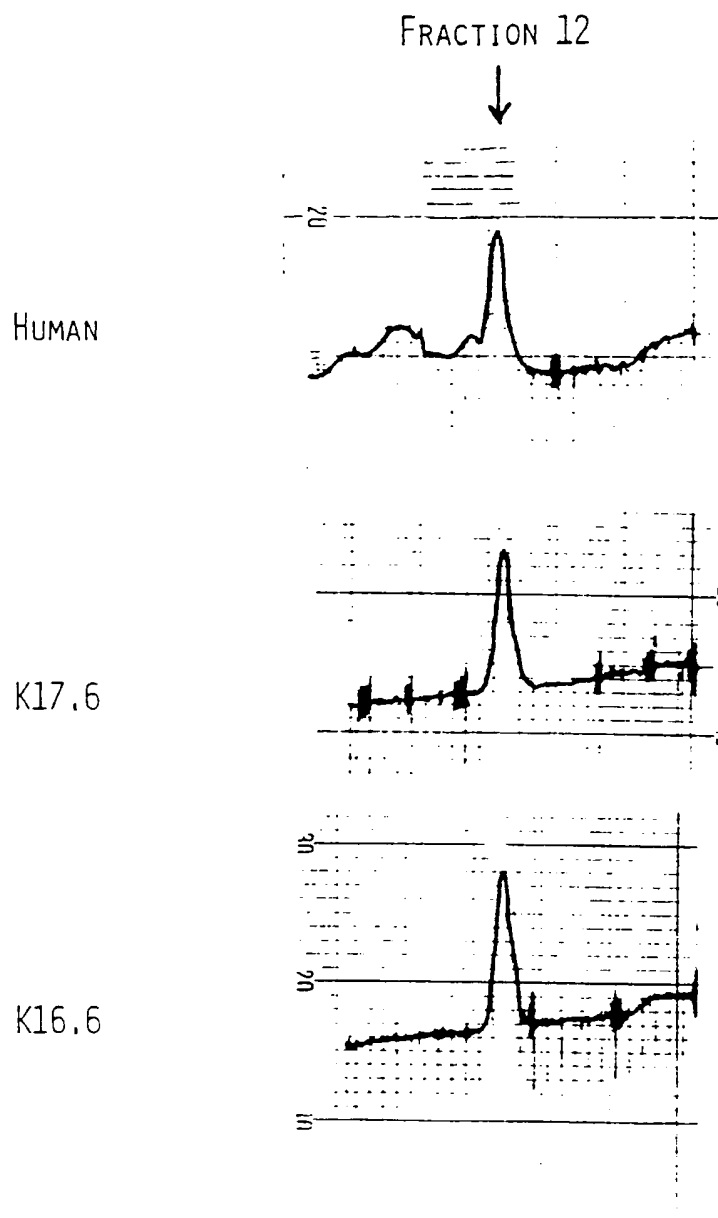


FIG. 9

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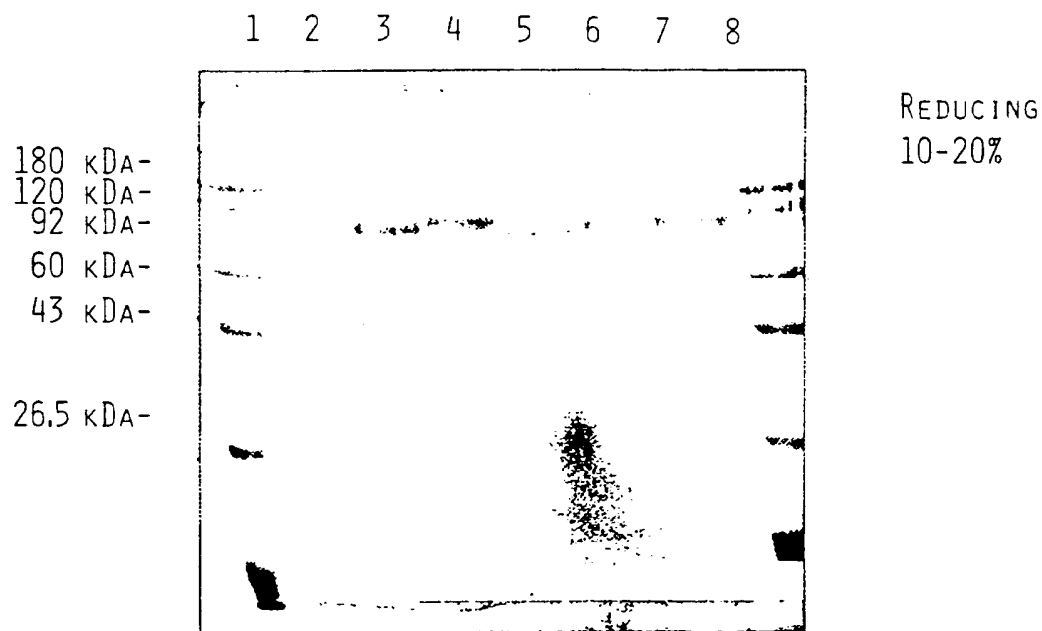


FIG. 10

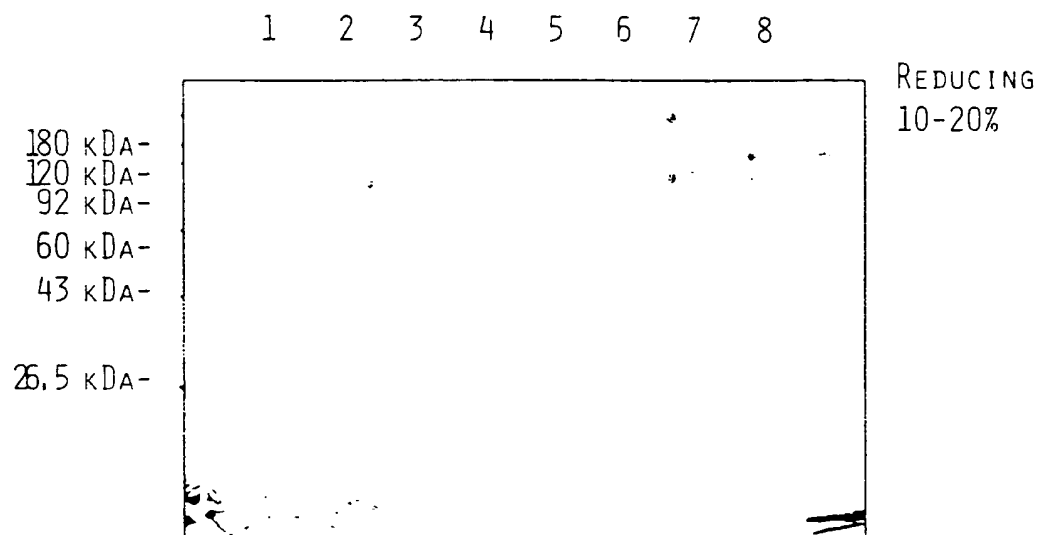


FIG. 11

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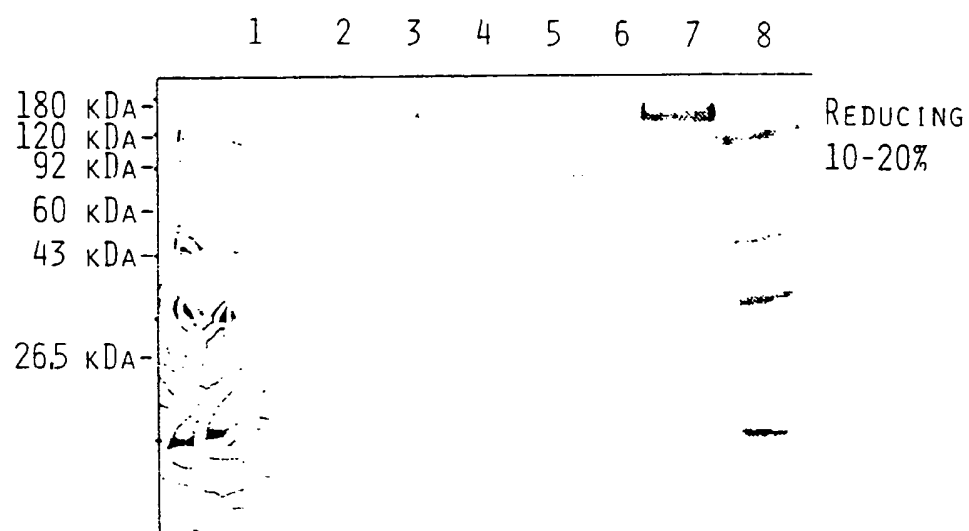


FIG. 12

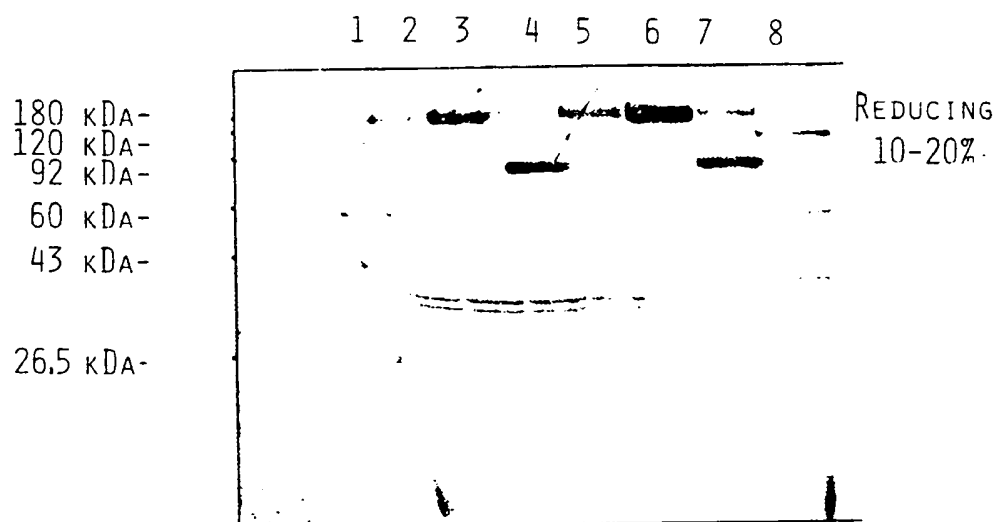


FIG. 13

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00225

| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup><br>According to International Patent Classification (IPC) or to both National Classification and IPC<br>IPC5: C 12 N 15/15, A 61 K 37/64, C 07 K 13/00, C 12 P 21/02                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                       |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
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| <b>II. FIELDS SEARCHED</b><br><div style="text-align: center; margin-top: 5px;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 25%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding: 5px;">IPC5</td> <td style="border: none; padding: 5px;">A 61 K; C 12 N; C 07 K</td> </tr> </table> <div style="text-align: center; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched<sup>8</sup></div> <p style="margin-top: 10px;">SE,DK,FI,NO classes as above</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                       |                                     | Classification System                                     | Classification Symbols                                                                                         | IPC5                                | A 61 K; C 12 N; C 07 K |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| Classification System                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Classification Symbols                                                                                                                                                                                                                                                                |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| IPC5                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | A 61 K; C 12 N; C 07 K                                                                                                                                                                                                                                                                |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category *</th> <th style="width: 60%;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;">Proc Natl Acad Sci USA, Vol. 82, No. 8, 1985 Kan, Chen Chen et al.: "Nucleotide sequence of cDNA encoding human alpha-2-macroglobulin and assignment of the chromosomal locus", see page 2282 - page 2286</td> <td style="vertical-align: top;">1,2,4, 14-28, 36-50, 52-59</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="text-align: center; vertical-align: top;">--</td> <td style="vertical-align: top;">5-10, 29-34</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Chemical Abstracts, volume 96, no. 15, 12 April 1982, (Columbus, Ohio, US), Mortensen, Steen Bet al.: "Primary and secondary cleavage sites in the bait region of alpha-2-macroglobulin ", see page 253, abstract 117756z, &amp; FEBS Lett 1981, 135( 2), 295-300</td> <td style="vertical-align: top;">5-10, 29-34</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">Chemical Abstracts, volume 95, no. 7, 17 August 1981, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of the 'bait' region for proteinases in alpha-2-macroglobulin. Nature of the complex ", see page 261, abstract 57059s, &amp; FEBS Lett 1981, 127( 2), 167-173</td> <td style="vertical-align: top;">1-59</td> </tr> </tbody> </table> <div style="margin-top: 10px;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div> </div> |                                                                                                                                                                                                                                                                                       |                                     | Category *                                                | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup> | Relevant to Claim No. <sup>13</sup> | X                      | Proc Natl Acad Sci USA, Vol. 82, No. 8, 1985 Kan, Chen Chen et al.: "Nucleotide sequence of cDNA encoding human alpha-2-macroglobulin and assignment of the chromosomal locus", see page 2282 - page 2286 | 1,2,4, 14-28, 36-50, 52-59      | Y                                                                                             | --                  | 5-10, 29-34 | Y | Chemical Abstracts, volume 96, no. 15, 12 April 1982, (Columbus, Ohio, US), Mortensen, Steen Bet al.: "Primary and secondary cleavage sites in the bait region of alpha-2-macroglobulin ", see page 253, abstract 117756z, & FEBS Lett 1981, 135( 2), 295-300 | 5-10, 29-34 | A | Chemical Abstracts, volume 95, no. 7, 17 August 1981, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of the 'bait' region for proteinases in alpha-2-macroglobulin. Nature of the complex ", see page 261, abstract 57059s, & FEBS Lett 1981, 127( 2), 167-173 | 1-59 |
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| A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Chemical Abstracts, volume 95, no. 7, 17 August 1981, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of the 'bait' region for proteinases in alpha-2-macroglobulin. Nature of the complex ", see page 261, abstract 57059s, & FEBS Lett 1981, 127( 2), 167-173 | 1-59                                |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| <b>IV. CERTIFICATION</b> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border: none;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border: none; padding: 5px;">11th December 1990</td> <td style="border: none; padding: 5px;">1990 -12- 13</td> </tr> <tr> <td style="border: none;">International Searching Authority</td> <td style="border: none;">Signature of Authorized Officer</td> </tr> <tr> <td style="border: none; text-align: center; padding: 10px;"> <b>SWEDISH PATENT OFFICE</b><br/> <small>Form PCT/ISA/210 (second sheet) (January 1985)</small> </td> <td style="border: none; padding: 5px;"> <br/>           Yvonne Siösteen         </td> </tr> </table>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                       |                                     | Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report                                                            | 11th December 1990                  | 1990 -12- 13           | International Searching Authority                                                                                                                                                                         | Signature of Authorized Officer | <b>SWEDISH PATENT OFFICE</b><br><small>Form PCT/ISA/210 (second sheet) (January 1985)</small> | <br>Yvonne Siösteen |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| Date of the Actual Completion of the International Search                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | Date of Mailing of this International Search Report                                                                                                                                                                                                                                   |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| 11th December 1990                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 1990 -12- 13                                                                                                                                                                                                                                                                          |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| International Searching Authority                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Signature of Authorized Officer                                                                                                                                                                                                                                                       |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |
| <b>SWEDISH PATENT OFFICE</b><br><small>Form PCT/ISA/210 (second sheet) (January 1985)</small>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | <br>Yvonne Siösteen                                                                                                                                                                                                                                                                   |                                     |                                                           |                                                                                                                |                                     |                        |                                                                                                                                                                                                           |                                 |                                                                                               |                     |             |   |                                                                                                                                                                                                                                                               |             |   |                                                                                                                                                                                                                                                                                       |      |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |                                                                                                                                                                                                                                                                                                                                                                                       |                      |
|----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------|
| Category *                                                                 | Citation of Document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                    | Relevant to Claim No |
| A                                                                          | Chemical Abstracts, volume 101, no. 11, 10 September 1984, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of human alpha-2-macroglobulin. V. The complete structure ", see page 237, abstract 85952p, & J. Biol. Chem. 1984, 259(13), 8318-8327<br>--                                                                                                          | 1-59                 |
| P                                                                          | Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), Sottrup-Jensen, Larset al.: "The alpha-macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha-macroglobulins ", see page 227, abstract 210722y, & J. Biol. chem. 1989, 264(27), 15781-15789<br>--                                    | 5-10,29-34           |
| P                                                                          | Chemical Abstracts, volume 112, no. 25, 18 June 1990, (Columbus, Ohio, US), Marynen, P et al.: "A genetic polymorphism in a functional domain of human pregnancy zone protein: the bait region. Genomic structure of the bait domains of human pregnancy zone protein and alpha-2-macroglobulin ", see page 167, abstract 230679p, & FEBS Lett. 1990, 262( 2), 349-352<br>--<br>----- | 5-10,29,34           |

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 90/00225

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on 90-11-01  
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| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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